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Studies in tentacle affinity chromatography

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Studies in Tentacle Affinity Chromatography

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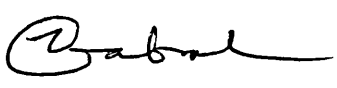

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ABSTRACT

A beaded co-polymer of 80% styrene and 20% divinylbenzene (average diameter 53 μm) was used as the starting material for the synthesis of a novel affinity support. A hydrophilic layer of polyvinyl alcohol (PVA) was adsorbed onto the surface, and stabilised by cross linking with terepthalaldehyde. Hydroxyethyl methacrylate was reacted with the PVA layer to introduce primary hydroxyl groups into the support surface. The support was activated with epichlorohydrin and the following Cibacron blue-polymer conjugates were immobilised to give a tentacle structure; dextran, hydroxyethyl starch, polyethylene imine, co-polymer of allyl alcohol and (N-hydroxymethyl)methyl acrylamide and polyvinyl alcohol. A small amount of dye conjugate was immobilised in all cases (<500 $\mu g\ ml^{-1}$), which led to very low protein binding capacities.

Underivitised dextran was immobilised to increase the amount of bound polymer, followed by reaction with Cibacron blue. The concentration of dye was varied in the coupling medium so as to produce four adsorbents with different dye loadings. The adsorptive performance was studied using the apparatus described by Horstmann *et al.*, (1986). There was a marked decrease in capacity with increased dye loading, the dissociation constant (K_d) was of the order 10^{-7} M and appeared independent of dye loading. The binding kinetics of the tentacle support were five times faster than a conventional porous polysaccharide support. The hydrodynamic performance was studied, and the tentacle adsorbent displayed the characteristics of a rigid support. The resolving power of the resin was tested using the purification of fumarase from crude chicken heart and rabbit liver extracts. The binding of the enzyme was poor to the tentacle support. The eluted protein was of low purity, although there was an increase in specific activity.

A polyhydroxyl containing monomer was synthesised; (N-[Tris(hydroxymethyl)methyl] methacrylamide) by reaction of methacrylic anhydride and Tris which was shown to be pure by nmr and mass spectroscopy. However, the yield of monomer obtained was low. Alternative synthetic reactions did not improve the monomer yield which was insufficient for polymer synthesis.

CONTENTS

Abbreviations

APS	ammonium persulphate
BSA	bovine serum albumin
DCC	dicyclohexyl carbodiimide
DMF	dimethyl formamide
DMSO	dimethyl sulphoxide
HE	hydroxyethyl
HEMA	2-hydroxyethyl methacrylate
K_d	dissociation constant
LDH	lactate dehydrogenase
PEI	polyethylene imine
PVA	polyvinyl alcohol
q_m	maximum binding capacity
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethylethylenediamine
TLC	thin layer chromatography
Tris	Tris(hydroxymethyl)aminomethane
U	enzyme units

Chapter One

1. INTRODUCTION

Advances in biotechnology have brought about the manufacture of many important products be they proteins or antibiotics; all of which require some degree of purification. The target application of the product determines the purity level required. In the case of the bulk quantities required for food and chemical industries, purity is of secondary importance to cost. However, proteins required for therapeutic use must be of the highest purity which is reflected in their high cost (Wheelwright 1989; Hammond and Scawen, 1989). The small quantities of therapeutic proteins required for use *in vivo* must have very low levels of nucleic acids, endotoxins, protein aggregates and leachates from column applications and other contaminating proteins (Spalding, 1991).

1.1. PRODUCTION OF PROTEINS

The source of the product is an important consideration and must be taken into account when planning a purification strategy. Animals are rarely used, mice for example can produce approximately 50 mg of antibody. However, scale-up requires a large number of animals; 20 000 mice may be needed to produce 1 kg of antibody (Birch *et al.*, 1985). Most proteins are purified from micro-organisms such as; *Escherichia coli, Saccharomyces cerevisiae and Bacillus subtillis*. Micro-organisms can be grown in very large quantities and fermentation can be carefully controlled so that there is little batch to batch variation (Stanbury and Whitaker, 1984). Gene cloning techniques enable the recombinant protein to be expressed as a large percentage of the total cell protein.

The host cell has to be chosen with care, proteins expressed in *E.coli* tend not to be secreted and aggregate into insoluble inclusion bodies. Inclusion bodies are relatively easy to purify, the inclusion bodies have to be denatured and the proteins have to be refolded back into their native state (Marston, 1989). Secreted proteins are easier to purify because the proteins are relatively free from contaminating proteins, and can be readily separated from the host cell (Palva *et al.*, 1983).

Bacillus subtillis could gain in popularity as a target for genetic modification because proteins are naturally secreted (Brock *et al.*, 1984). However, the genetics of *B. subtillis*

are not as well understood as those of *E. Coli.* This organism also secretes proteases which attack the recombinant product reducing the overall yield.

A drawback of prokaryote systems is their inability to glycosylate proteins, as would occur in the Golgi apparatus in eukaryote cells. *S.cerevisiae* is sometimes used because of the presence of a glycosylation pathway, although glycosylation is not as extensive as higher eukaryotes (Bradley, 1990). *S.cerevisiae* only secretes a small amount of protein and is notoriously difficult to break open to release cellular contents. Cultured animal cells such as; Chinese hamster ovary cells, xenopus oocytes and hybridomas cells are filling this void. Large proteins such as antibodies have been expressed with the correct amount of glycosylation (Harris, 1989). The major drawback of animal cells is that it is difficult to achieve high cell densities ($>10^7$ cells ml^{-1}; Spier, 1987). As a result there is a decrease in the amount of protein produced. There are also inherent difficulties in fermentation due to the low mechanical strength of animal cells (Emery *et al.*, 1987).

1.2. PROTEIN PURIFICATION

The first stage of downstream processing is the recovery of the protein from the cell fermentation process. Multiple steps are usually required with consequent loss of product at each stage (Fish and Lilly, 1984). Thus a judicious choice of unit operations is required to achieve a good product yield.

Centrifugation or microfiltration are used to separate the cells from excreted proteins. Non secreted proteins or inclusion bodies are extracted by cell disruption techniques, such as ball mills, solid/liquid extrusion or digestion of the cell wall followed by osmotic shock. The protein is now in a soluble form and free of large particulates. Nucleic acids are removed by the actions of nucleases or precipitated by polyethylene imine (Brewer and Sassenfeld, 1989).

The second step is to isolate the target protein to a high level of purity. One of the best tools for achieving this goal is the use of biospecific affinity chromatography. This technique exploits the specific interactions of biological macromolecules with their ligands (substrates, inhibitors, antigens, sugars and nucleic acids). The interactions are reversible, attachment of the specific ligand to an insoluble support and the target protein would bind specifically to the ligand whilst other proteins pass through unretarded (figure 1.1). The target protein is eluted by altering the protein ligand interaction. This

can be achieved by either bio-specific elution (substrates, inhibitors or cofactors) or non-specific elution (change of salt concentration, pH, ionic strength, temperature or polarity). Dilute proteins can be concentrated from large volumes of fermentation broth. Thus biospecific affinity chromatography can potentially purify proteins to a high level purity in a single step (Sii and Sadana, 1991).

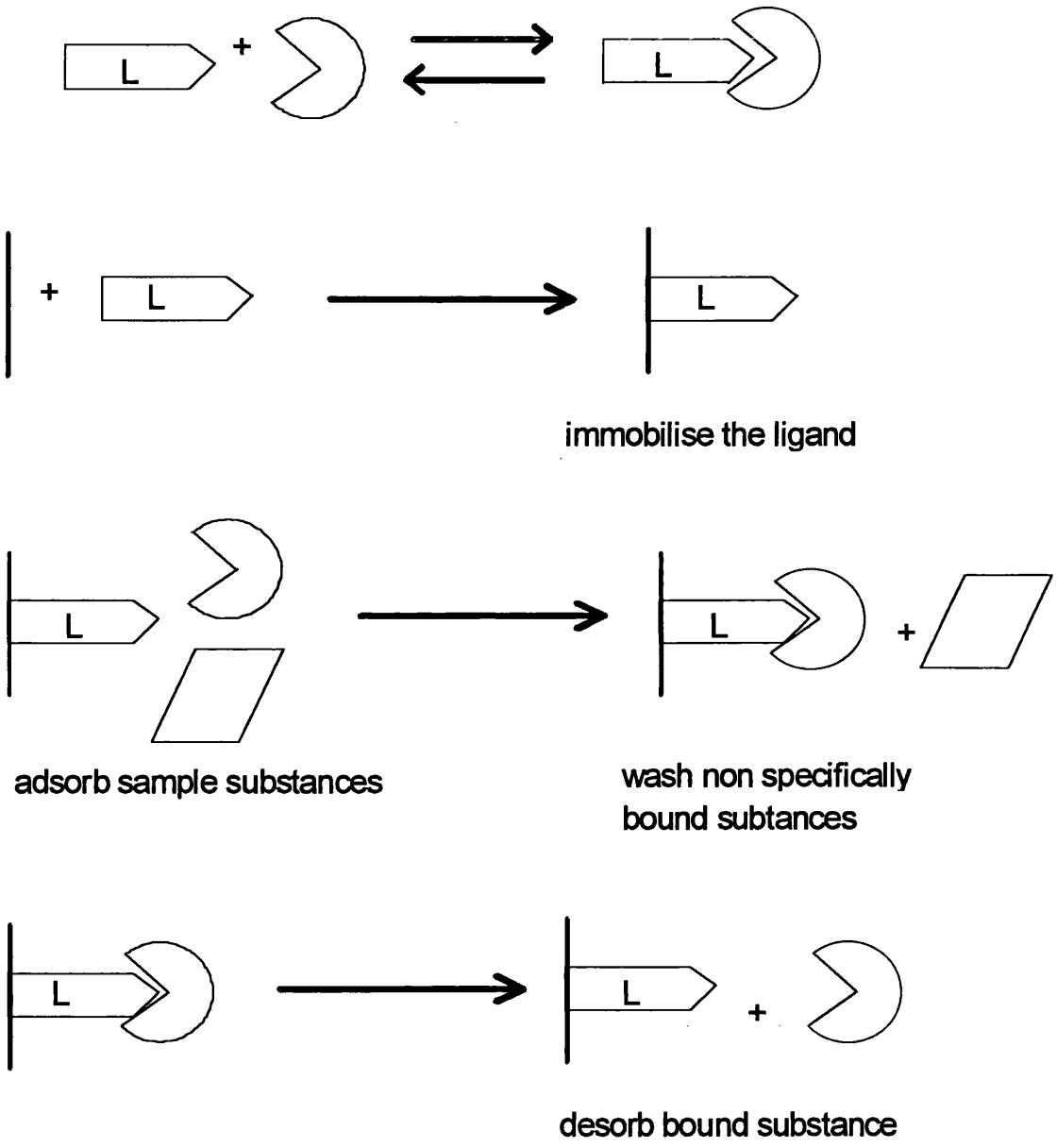


Figure 1.1: *Principle of affinity chromatography*

1.3. AFFINITY CHROMATOGRAPHY-a brief history

The first application of affinity chromatography was the selective adsorption of amylase onto insoluble starch by Starkenstein (1910). This technique was improved upon by Lerman (1953) who immobilised *p*-azobenzenearsonate onto cellulose powder. The adsorbent was succesfully used to isolate antibodies from whole serum. The advent of beaded agarose (Hjerten, 1964) meant that a more readily chemically derivitisable support was available.

In 1967 Axen and co-workers found that molecules containing amino groups could be immobilised onto agarose using cyanogen bromide. This new chemical technique allowed a wide range of affinity ligands to be immobilised. However, the isourea linkages formed from the cyanogen bromide activation were prone to a slow steady hydrolysis. The isourea linkage carries a positive charge at neutral pH (Yang and Tsao, 1982). Alternative activation chemistries were developed:

- Bisepoxides (Sundberg and Porath, 1974);
- Tesyl and tosyl chloride (Nilson and Mosbach, 1980);
- Periodate oxidation (Junowicz and Charm, 1976);
- 2-Fluoro-1-methylpyridinium salts (Ngo, 1986);
- Cyanuric chloride (Biagioni, 1978);
- Carbonyldiimidazole (Bethell *et al.*, 1979);
- Carbodiimides (Lowe and Dean, 1974).

The linkages formed by these chemistries have greater chemical stability and are also uncharged (Parikh and Cuatrecasas, 1993). All the above mentioned coupling chemistries rely on the ligand containing nuclephiles; amino, thiol and hydroxyl groups. Another method which is beginning to find use is the Mannich reaction (Hermanson *et al.*, 1992) which is very useful for binding ligands that do not possess suitable nucleophiles. This is especially useful for coupling steroidal type ligands.

Affinity chromatography has been developing empirically, and is being increasingly used for industrial process applications (Knight, 1989). A greater understanding of the process constraints is required so that scale up can be carried out successfully.

There are various types of affinity chromatography:

- Immunoaffinity chromatography;
- Metal chelate affinity chromatography;
- Covalent affinity chromatography;
- Hydrophobic interaction chromatography;
- Group specific affinity chromatography.

Group specific affinity chromatography will be concentrated upon, because of the relevance to the experimental work which will be presented in later chapters. One of the main drawbacks of affinity chromatography is that the ligands tend to be chemically and biologically labile. Thus adsorbents would have to be replaced regularly increasing process costs. Another consideration is that every target protein would need its own specific ligand which on the process scale may prove to be too expensive.

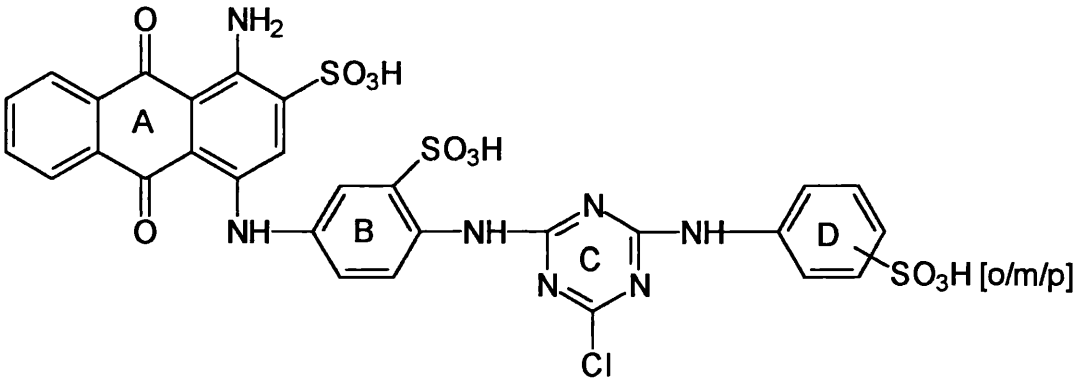
Group specific ligands are now being used to solve this problem. Protein A (*Stapthylococcus aureus*) and protein G (*Streptococcus sp.*) bind the Fc regions of different classes of mammalian IgG antibody (Horstmann and Chase, 1989, Akerstrom and Bjorck, 1986). A chimeric protein A/G has been developed which has a greater specificity for immunoglobulins, than either parent protein A or G (Elliason *et al.*, 1988). Protein A/G adsorbents are useful because they orientate the antibody in a "favourable" position, such that both binding sites are available (Gyka et al., 1983). Immobilised nucleotides such as 5' AMP binds NAD^+ dependent dehydrogenases and ATP dependent kinases. 2'5' ADP binds $NAD(P^+)$ dependent dehydrogenases and other enzymes which require $NAD(P^+)$. Lectins, such as concanavalin A, will bind molecules which contain α-D-glucopyranosyl and α-D-mannopyranosyl. Although these ligands are efficient they all have several drawbacks in terms of cost, ligand utilisation, biological and chemical stability.

1.4 Biomimetic Ligands

Biomimetic ligands or pseudo ligands are group specific ligands which mimic the binding of their natural counterparts. An example of these ligands are the triazine dyes. This discovery was first reported by Haeckel (1968) and co-workers during the purification of yeast pyruvate kinase. The enzyme co-eluted with the void volume column marker blue dextran during gel permeation experiments. The chromophore responsible for this phenomenon is the triazine dye Cibacron blue F3GA (Figure 1.2) which was used to colour the dextran.

The reactive triazine dyes were introduced by ICI in 1954 and were designed for textile applications (Dean and Qadri, 1983). The dyes all have a mono or di chlorine triazine ring which is used to attach the dye permanently to polymers such as cotton and wool.

Early attempts to use blue dextran as a purification aid were laborious and time consuming due to a two step purification procedure. The first step was the isolation of the soluble blue dextran-enzyme complex from contaminating proteins by gel filtration chromatography. A second gel filtration step was required at high ionic strength to separate blue dextran from the protein.



Cibacron Blue F3GA (Procion Blue HB)

Figure 1.2: *The structure of Cibacron blue F3G-A: where A is the anthraquinone ring, B is the diaminobenzene sulphonate ring, C is the triazine ring and D is the terminal ring.*

Ryan and Vestling (1974) devised a method of immobilising blue dextran onto an insoluble matrix which was used for the purification of lactate dehydrogenase from rat tissues. However, the binding capacity of the blue dextran column was low, and it was discovered that dextran was inert. The chromophore itself was immobilised to produce a specific adsorbent (Bohme *et al.*, 1972; Heyns and De Moor, 1974).

Early theories stated that the dye acted as a conformational probe since it was found that the dye was able to bind at the co-factor binding site of dehydrogenases and kinases (Bhome *et al*, 1972; Thompson *et al.*, 1975). This binding site was termed the "dinucleotide fold" and is conserved in many enzymes requiring adenyl co-factors

(NAD(P^+), ATP and CoA). The dinucleotide fold consists of about 120 amino acids arranged in a five or six β-sheet core connected by α-helical intra strand loops located above and below the β-sheet (Rossman *et al.*, 1974). These theories were given greater credibility when it was discovered that the dye-enzyme complexes could be dissociated by high salt concentrations or low concentrations of the respective co-factors (ATP and NAD).

Structural analogues of the dye showed that only rings A and B where involved in protein binding (figure 1.2, Beissner and Rudolph, 1978; Biellmann *et al.*, 1982). This is illustrated in figure 1.3 where the dye molecule is superimposed on the NAD co-factor binding site (Lowe *et al.*, 1992). The terminal rings of the dye molecule (rings c and d, figure 1.2) are too rigid to overlap fully in the co-factor binding site (Lowe *et al.*, 1986).

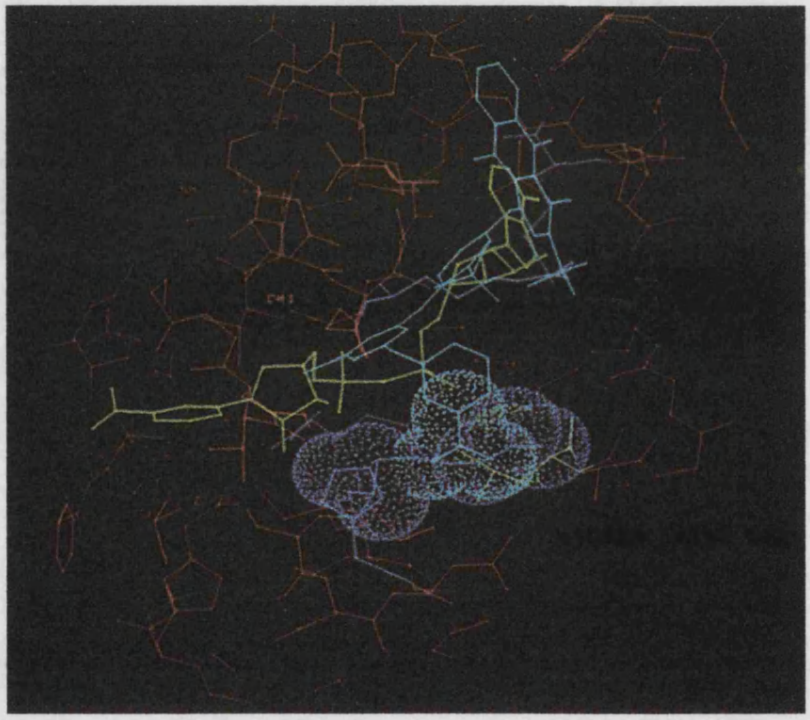


Figure 1.3: *Cibacron blue F3GA (blue) superimposed on the NAD cofactor binding site (green) of alcohol dehydrogenase (Lowe et al, 1992).*

The reason for the close similarity in binding is illustrated in figure 1.4 where comparison of Corey-Pauling-Koltin (CPK) models of NAD and the dye molecule shows that there is a remarkable similarity between the two molecules. Spectrophotometric, kinetic and chromatographic studies led Thompson and Stellwagen (1976) to predict that:

- Most NAD^+ dependent dehydrogenases and ATP dependent kinases interact with Cibacron blue;
- The interaction is competitive with respect to the corresponding nucleotide substrate;
- The dye-enzyme complexes can be cleaved either specifically with a low concentration of the respective co-factor, or unspecifically with a high concentration of salt;
- The complex formed between the enzyme and the dye causes a shift in the visible spectrum of the dye, which can be used to monitor the dye -enzyme interaction;
- The binding site is evidently located in a highly apolar region of the enzyme.

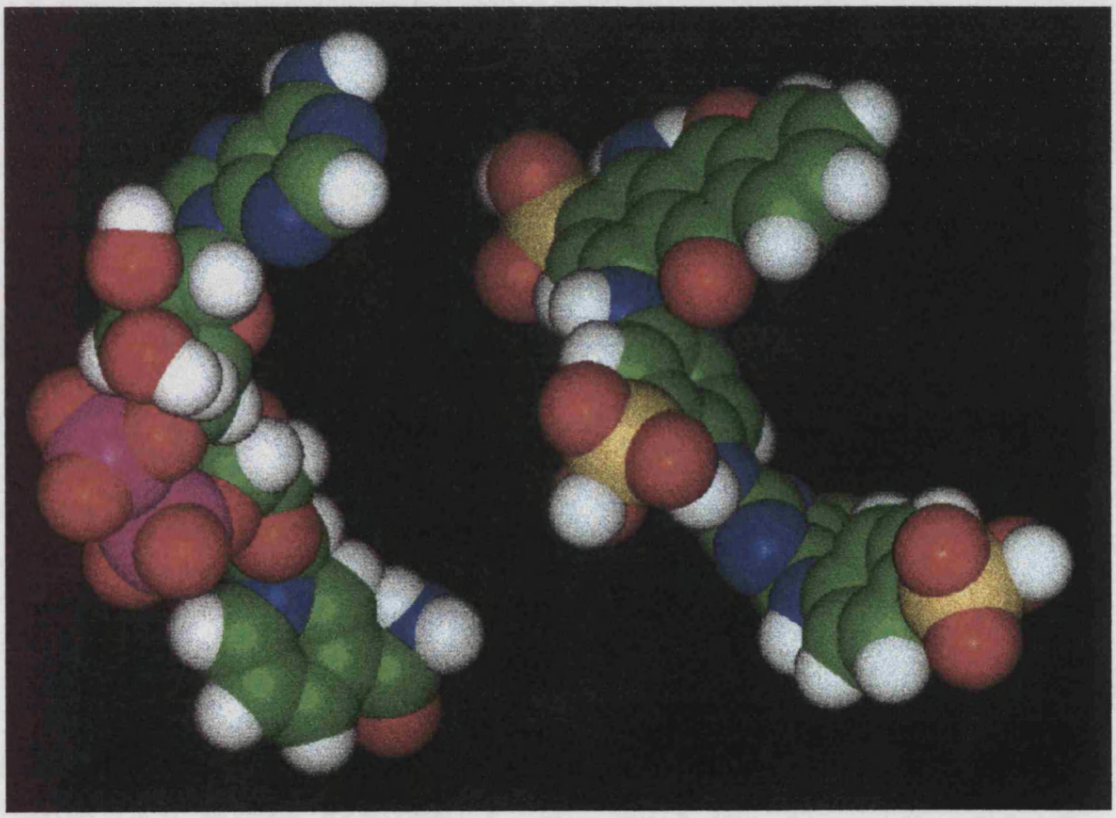


Figure 1.4: *Corey-Pauling-Koltin (CPK) structural model of NAD and Cibacron Blue F3GA.* NAD is shown on the left in the conformation of binding to dehydrogenases. Cibacron blue F3GA model is shown on the right in a conformation similar to the NAD. Atom key: carbon (green), nitrogen (blue), oxygen (red), sulphur (yellow), phosphate (magenta) and hydrogen (white).

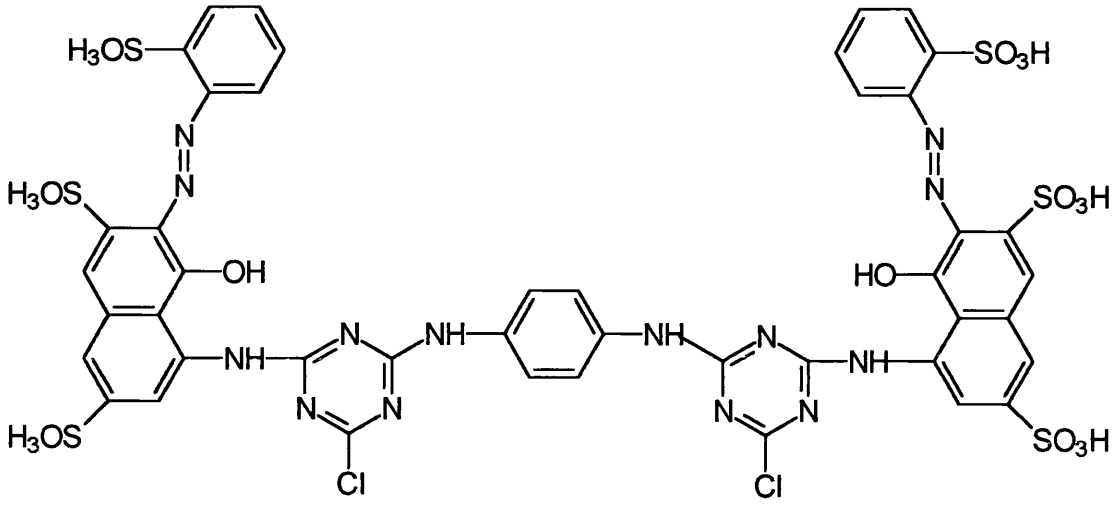
However, the conformational probe theory of Thompson and Stellwagen was flawed. Proteins which possessed the "dinucleotide fold" did not interact with the dye (Dean and Watson, 1979). It also became apparent that Cibacron blue was able to interact strongly with a wide variety of proteins which do not contain the dinucleotide fold as outlined in table 1.1.

Table 1.1: Proteins purified by Cibacron blue

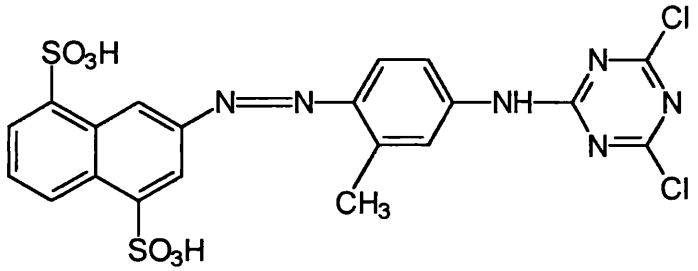
Protein	Affinity ligand previously used	Reference
Albumin	-	Travis *et al* 1976
Interferon	L-tryptophan or Concanavalin A	Jankowski *et al* 1976
Blood clotting factor X	-	Vician and Tishkoff 1976
Choline acetyltransferase	Coenzyme A	Roskoski *et al* 1975
Isoleucyl tRNA synthetase	L-isoleucinyl-5'-adenyalte	Moe and Piszkiewicz 1979
Restriction endonuclease	-	Baksi *et al* 1978

The introduction of Procion red HE-3B (figure 1.5) as a dye ligand also destabilised Thompson and Stellwagen's hypothesis because the dye did not structurally resemble NAD, ATP or even Cibacron blue. Despite these facts Procion red is a competitive inhibitor of dehydrogenases and kinases. Enzymes were also eluted from Procion red dye columns with low concentrations of co-factor or high salt concentration. In fact it became apparent that Procion red interacted more strongly with $NADP^+$ dependent dehydrogenases whilst Cibacron blue interacted more strongly with NAD dependent dehydrogenases (Watson *et al.*, 1978).

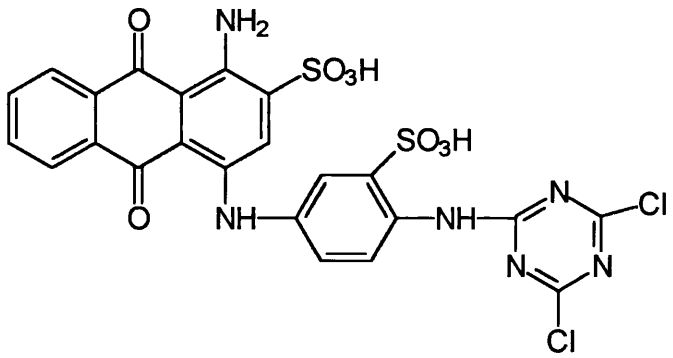
Other triazine dyes have been discovered which interact with proteins. Procion yellow MXR (figure 1.5) is used for the purification of pyruvate kinase (Makriyannis and Clonis, 1993) and inosine 5'-monophosphate dehydrogenase (Lowe *et al.*, 1980). There are now such a great number of textile dyes which interact with proteins that a rapid screening system has been designed by Scopes (1986) to enable the selection of the most appropriate dye ligand.



Procion Red HE3B



Procion Yellow MXR



Procion Blue MXR

Figure 1.5: *Structures of triazine dyes commonly used for affinity separations*

The versatility of these biomimetic ligands has led Koppersclager *et al.* (1982) to propose the following;

- The dye binding site is an apolar region in the surface of the protein molecule surrounded by hydrophilic amino acid residues;
- Hydrophobic as well as electrostatic forces mainly contribute to the stability of the complex;

- The dinucleotide binding fold is a special case of the dye binding sites;
- In principle, all ionic aromatic compounds are capable of binding to proteins which possess a hydrophobic pocket in their surface which is sufficiently large for accommodation; an apparent specificity is achieved by larger ring systems since only a few proteins possess apolar regions;
- The better the arrangement of the ionic groups of the dye fits to the arrangement of the corresponding functional groups of the protein, the stronger the complex binding.

The versatility of the dyes is so great that dyes can be used subtractively such that contaminating proteins are bound whilst the target species will pass through the dye column unretarded. Albumin is removed from plasma, enabling the albumin depleted plasma to be resolved into discrete components (Travis *et al.*, 1976).

Table 1.2 illustrates the advantages of triazine dyes over conventional biological ligands. Provided the protein of interest has a reasonably high affinity for the dye, a high level of purity can be achieved with low process costs (Scopes, 1987).

Table 1.2: Comparison of immobilised 'biomimetic' dyes with conventional adsorbents

Criterion	Immobilised 'biomimetic' dyes	Conventional biospecific adsorbents
cost	inexpensive, commodity chemicals	can be expensive
specificity	moderate	moderate to high
Synthesis of adsorbents	facile, often one step reaction	lengthy synthetic route and toxic activation reagents
capacity	high (>10% ligand utilisation)	low ligand utilisation (0.01-10%)
scale up potential	virtually limitless	very limited
re-usability	very high	low
stability	chemically and biologically stable	tend to be labile
sterilizability	high	mostly low

1.5. SPACER ARM

The spacer arm or leash is a low molecular weight group which separates the ligand from the matrix backbone which can enhance the binding interaction with the target protein. The use of a spacer arm was introduced by Cuatreacasas (1970) and Steers (1971). Lowe and co-workers (1973) found that a directly coupled ligand may not be able to interact with a specific molecule because of the binding site being buried deep in the protein surface. Thus the binding interaction will be either greatly weakened or binding may not take place at all. The spacer molecule is very useful for projecting small ligands away from the matrix surface and giving the ligand greater flexibility such that the correct orientation is favourable. The classic example is the purification of β-galactosidase. Steers and co-workers (1971) showed that a spacer arm of 10 Å (1 nm) was not sufficient to improve binding of β-galactosidase; a spacer of over 20Å was required to achieve binding. The binding interaction was found to be so strong that

elution with the ligand was not effective. A pH of 10 was required to effect elution. This was thought to be due to the presence of the spacer arm. However, the findings were questioned and it was found that the enhanced binding was due to non-specific interactions from the spacer. O'Cara *et al* (1973) found that when carbon spacers were attached without any ligand the binding was very similar to that observed by Steers et al (1971). The optimum spacer length was found to be C_6 by and C_6-C_8 by Lowe, although smaller molecules can still be useful as spacer molecules (figure 1.6).

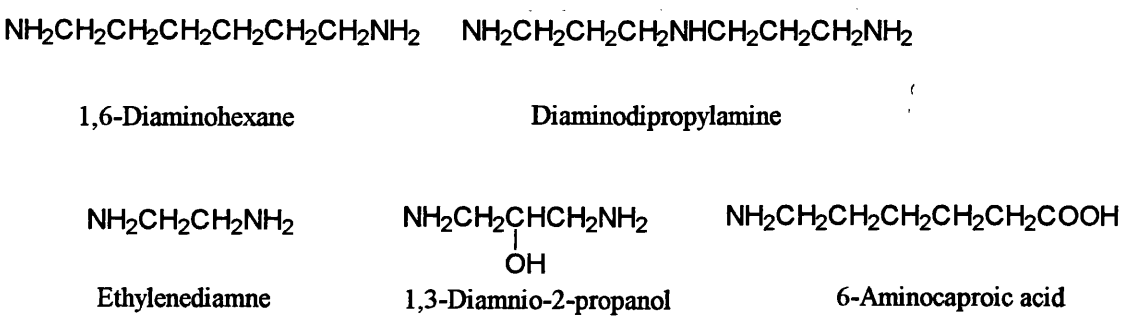


***Figure 1.6:** Commonly used spacer molecules*

Spacer molecules are useful for orientating the ligand in the correct conformation because the coupling chemistry can be designed so that specific groups such as thiols, hydroxyls, carbohydrates or active hydrogen (Mannich reaction) on the ligand can be targeted. Thus groups involved with ligand binding are not used for immobilisation which ensures a level of control when synthesising an adsorbent.

Another consideration when using the spacer molecule is to reduce the amount of non-specific binding due to the hydrophobic methylene backbone. A number of measures can be taken to alleviate this problem:

- A large excess of ligand in the coupling medium to ensure that the majority of the spacer molecules have reacted with ligand;
- The use of spacers with secondary amines, ethers, hydroxyls or amide bonds which increase the hydrophilic character of the spacer reducing non-specific interactions;
- Controlling the amount of activation so as to reduce the amount of spacer actually introduced into the matrix;
- Spacers, which carry a charge at the end of the spacer for example 1,6 diamino hexane will carry a positive charge at neutral pH, and will introduce ionic character into the matrix, thus it is imperative to cap such groups with a non ionic molecule.

1.6. THE MATRIX

For successful protein purification the support matrix must meet the following requirements:

- The matrix should be insoluble and hydrophilic;
- Possess a large internal surface area for protein binding to occur;
- Easily derivatisable for ligand immobilisation;
- Display low non-specific binding of proteins;
- Good mechanical stability to minimise flow resistances;
- Uniformly porous with pores large enough to permit unhindered diffusion of macromolecules (this only true for porous matrices);
- Chemically and biologically stable;
- Withstand cleaning in place procedures.

At present there is not a matrix which fits all the above criteria. The matrix chosen is usually the best compromise available for a particular application.

1.6.1. Agarose

The most commonly used matrix is cross linked agarose (Sepharose®) which fits most of the above criteria except for its susceptibility to microbial growth (Scawen and Hammond, 1989) and lack of mechanical strength. Agarose is a polysaccharide based on polymeric chains of the disaccharide agarobiose (D-galactose and 3,6-anhydro-1-galactose) and is obtained from seaweed. The beads form spontaneously from the molten form of agarose to yield a highly porous support (Hjerten, 1964).

However, the bead structure is mechanically very weak and is unstable above 40°C and in the presence of denaturants such as guanidine hydrochloride (Pharmacia). These factors were overcome by cross linking agarose with 2,3-dibromopropanol or epichlorohydrin (figure 1.7, Porath *et al.*, 1971). Cross linking of the support enhances the structural stability of the beads such that the beads tolerate extremes of pH, temperature, denaturing agents and organic solvents. Conventional crosslinked beads (Sepharose CL) are ideal for laboratory scale purification; however the lack of resistance to high pressures and flow rates precludes its use for industrial applications.

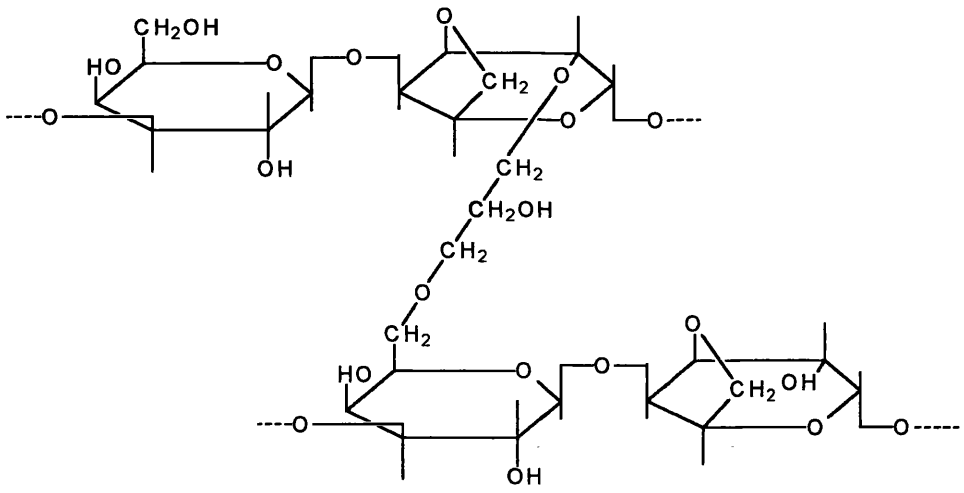


Figure 1.7: Partial structure of cross linked agarose (Sepharose CL®)

1.6.2. Dextran

Dextran is a polysaccharide produced by the bacterium *Leuconostic mesenteroides*, consisting of glucose molecules linked by α-1,6 bonds. The polymer is primarily a straight chain with a few α-1,3 branches of about 40 residues (Sidebotham, 1974). The bead structure is formed (Sephadex) by cross linking with epichlorohydrin (figure 1.8). The degree of cross linking determines the porosity of the support (Porath and Flodin, 1959). However, the beads which are the most porous and hence suitable for affinity separations, are easily compressed and swell considerably in aqueous environments. Cross linked agarose (Sepharose CL) is by far a superior support for affinity separations than dextran.

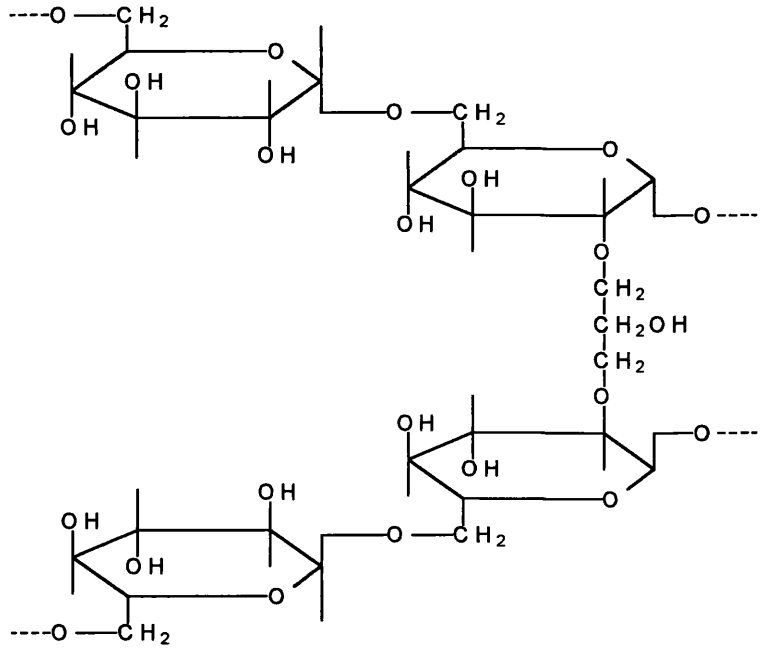


Figure 1.8: Partial structure of cross linked dextran (Sephadex®)

1.6.3. Cellulose

Cellulose is a polysaccharide based on glucose molecules linked by β-1,4 bonds. The polymeric structure is a long straight chain (figure 1.9). Hydrogen bonding cause the chains to form fibrils. The fibrils are so tightly bound that cellulose has virtually no porous structure or increased surface area. The structure is only disrupted in the presence of strong acids, alkalis and oxidising agents. A more porous structure can be formed by treating cellulose with 0.5 M NaOH which disrupts the hydrogen bonding. Cellulose is available in fibrous, granular and bead forms. However the support cannot withstand high flowrates and the capacity is about a tenth of conventional supports (Hermanson *et al.*, 1992a).

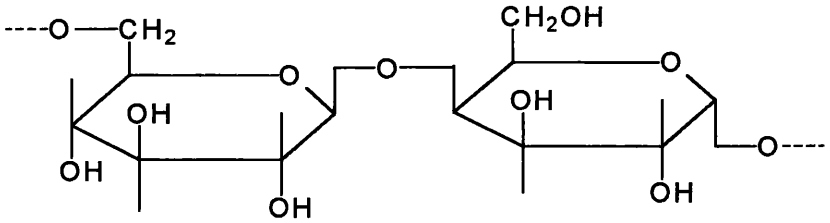


Figure 1.9: Partial structure of cellulose

1.6.4. Silica

Inorganic matrices based on silica are mechanically stable, and have very good flow characteristics. Silica in its native state is acidic and denatures proteins (figure 1.10). Hydrophilic coating with silane or polysaccharides such as dextran or agarose (Chang *et al.*, 1976; Girot *et al.*, 1990) negates this non-specific interaction. The limitation with silica is the unstability above pH 8.0 (Roe, 1989) and non-specific binding of proteins (Girot *et al.*, 1990). The high mechanical stability makes silica a suitable matrix for HPLC applications. Affinity ligands have been attached to silica and a new type of affinity separations termed high pressure affinity chromatography (Chang *et al.*, 1976) has resulted.

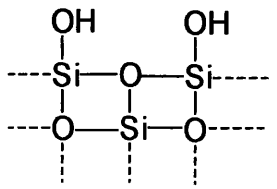
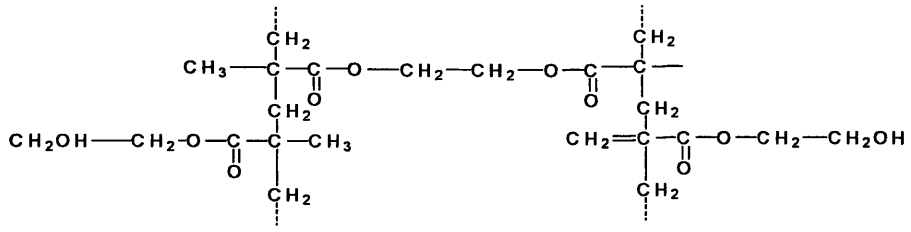


Figure 1.10: Structure of silica

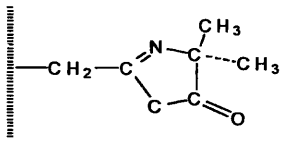
1.6.5. Synthetic supports

Some of the first synthetic matrices were synthesised by Hjerten and Mosbach (1962). The matrix was based on polyacrylamide which was a copolymer of acrylamide and cross linking agent N, N' methylene-bis(acrylamide). The matrix was chemically and biologically stable but lacked mechanically strength. The beads become increasingly compressible as the pore size increases (Roe, 1989); thus composites of these materials and polysaccharides dextran or agarose have been prepared (Sephacryl® and Superose® respectively). These supports combine the benefits of agarose/dextran but give the support added mechanical strength with the incorporation of polyacrylamide. These supports unfortunately have a reduced porosity when compared to the parent agarose or dextran.

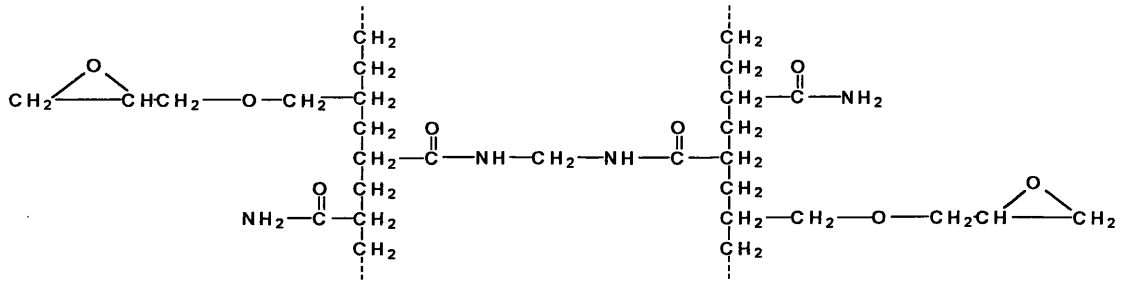
Subsequently a newer generation of synthetic supports has been developed which is primarily for gel filtration applications but can be used for affinity separations. These supports have superior chemical, biological and mechanical stability. In addition, the supports are stable to extremes of pH. Examples of these supports are; Toyopearl®, Azalactone®, Eupergit®, Trisacryl® and HEMA® (figure 1.11). The only draw back is after repeated cleaning and regeneration procedures, monomers may slowly leach with time. This limits the use of synthetic polymeric supports in the food and pharmaceutical industries (Roe, 1989).



HEMA



Azalctone

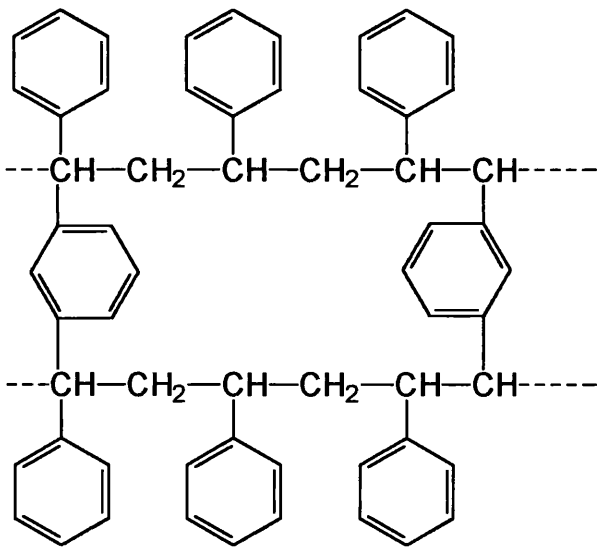


Eupergit

Figure 1.11: *Partial structures of the new generation of synthetic supports*

1.6.6. Polystyrene

Polystyrene is a co-polymer of linear styrene and cross linked with divinyl benzene (figure 1.12) and has been used in beaded form for various applications, such as waste water treatment, plates for ELISA's (Engvall and Prealmann, 1971), immunoassays and ion exchangers. Dowex® ion exchangers, manufactured by Biorad, have found widespread use for the purification of chemicals and for water softening procedures by removing ions. However the hydrophobic nature of the base matrix prevents the use of these ion exchangers for the purification of proteins (Roe, 1989). Polystyrene is extremely chemically stable as a result many classical organic reactions can be carried out such as nitration which requires a mixture of fuming concentrated nitric and sulphuric acids (Stark, 1974). The porosity of the beads is entirely dependent on the amount of crosslinker present (divinyl benzene). A polystyrene support with 4% divinyl benzene has an exclusion limit of 50-34 kD, whilst a support with 12% divinyl benzene will have an exclusion limit of 400 Daltons (Biorad). The degree of cross linking also determines the swelling characteristics of polystyrene in organic solvents (Biorad).



Polystyrene

Figure 1.12: *Structure of polystyrene*

Polystyrene is also used as the stationary phase for peptide synthesis; the chemical stability is such that the peptide can be synthesised in situ. Protective groups are cleaved off and the peptide can be eluted off the column without any detriment to the support (Atherton and Shepppard, 1989).

The ability to passively adsorb proteins has led to polystyrene particles (<1 μm) being used for agglutination tests in diagnostic kits. A new method of bead separation was also invented by encapsulating a paramagnetic iron core with polystyrene (Dynal, 1996). The beads can be isolated from the bulk media by application of a magnetic field. These beads are extremely useful tools for labelling of cells or for molecular biology as templates.

1.6.7. New advances in matrix technology

A major step forward in support matrix technology was the bead polymerisation technique developed by Ugelstad and co-workers (1983) which is based on the controlled swelling of sub micron particles in the presence of monomer. This method gives practically monodisperse beads of any diameter from approximately 1-100 μm. Commercially available beads using this technology are supplied with a diameter of 10-20μm with controlled porosity (Ellingson *et al.*, 1990). These materials are based on methacrylate or styrene/divinyl benzene monomers.

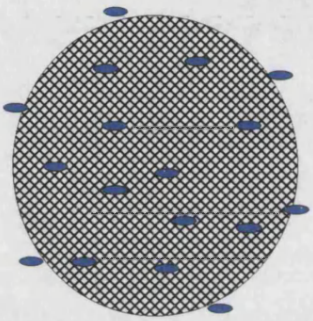
A new concept in column chromatography began with a support developed by Afeyan and co-workers (Poros®, 1990). The Poros support is based on a co-polymer of styrene/divinylbenzene. The bead is coated with a stable proprietary hydrophilic coating which can be derivitised for ligand immobilisation and is mechanically and chemically stable. The beads have a unique structure with large through pores (600-800 nm) which traverse the length of a bead and interconnecting diffusive pores (80-150 nm) which transfer fluid rapidly from the through pores throughout the bead (perfusion chromatography, Afeyan *et al.*, 1991).

Perfusion chromatography offers advantages over conventional porous supports such as cross linked Sepharose. Connective flow only occurs between the particle spaces and access to the immobilised ligand depends on molecular diffusive processes. Conversely, the large through pores allow connective flow through the individual bead in the Poros support. Once the mobile phase reaches the internal bead structure it is rapidly transferred into the diffusive pores, thus increasing the speed at which fluid is transferred. The advantage that the perfusion system offers is that at high linear flow rates there is no reduction in the capacity or resolution of the support. At present these matrices are available in small bead diameters (8-10 μm and 15-25 μm) which makes the support useful for HPLC applications. However, suitable bead diameters are not available for low pressure applications (50-300 μm). Another drawback is that the support is very expensive and a larger bead would probably increase the expense therefore making scale up doubtful.

1.7. Research Aims

Affinity chromatography is usually operated in one of two modes, porous and non-porous. Porous supports based on polysaccharides (Sepharose and Dextran) tend to have large internal surface areas for high protein binding capacities and are mechanically weak. Porous supports have pore diffusion limitations which result in slow binding kinetics (Chase, 1984). Conversely non-porous supports have high mechanical stability, exhibit good flow characteristics and exhibit fast binding kinetics (Anspach *et al.*, 1989). The protein binding capacity however is limited by the available external surface area which is a function of particle diameter. Pressure drop limitations put constraints on the minimum particle size, thus reducing the effective capacity of the beads (figure 1.13).

A compromise between these two extremes is the "tentacle affinity approach" (figure 1.13). Ligands are immobilised on to hydrophilic polymers which are attached to stable non-porous beads. The resulting support should show binding kinetics, mechanical and flow properties approaching that of non-porous supports; and have binding capacities approaching that of porous supports. This tentacle approach has been limited to ion exchange (Muller, 1990 and Tsuneda *et al.*, 1994) and metal chelate affinity applications (Hansson and Kagedal, 1981).



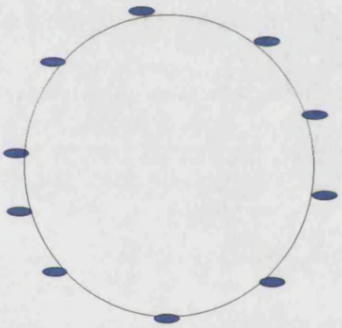
Porous Support

High capacity

Slow kinetics

Pore diffusion limitations

Mechanically weak



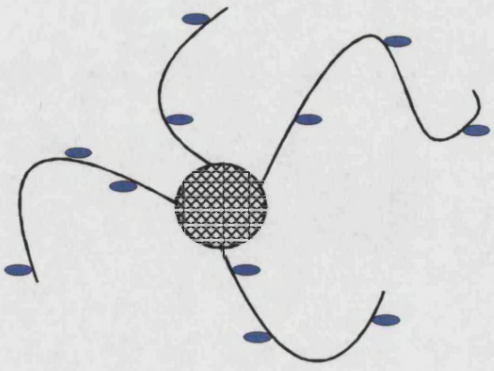
Non Porous Support

Low capacity

Fast kinetics

Pressure drop limitations

Mechanically strong



Tentacle Ligand Support

Tentacle approach

High capacity

Fast kinetics

Medium pressure drop

Mechanically strong

Figure 1.13: *Comparison between conventional affinity chromatographic supports and the tentacle affinity support*

Thus the aim of the project is to synthesise tentacle affinity supports with a range of ligand density and polymer molecular weights. The ligand will be the biomimetic Cibacron blue and the model proteins to be studied will be lysozyme and lactate dehydrogenase.

Cibacron blue will be immobilised on a variety of water soluble polymers of different molecular weight. The modified polymers will be fully characterised in terms of their protein binding properties using spectrophotometric titrations and stopped flow techniques. Once the modified polymers have been fully characterised the polymers will be immobilised onto a non-porous support. Thus forming a composite resin which has the ligands presented away from the support surface in a tentacle formation. The support matrix for the tentacle affinity adsorbent is a co-polymer of 80% styrene and 20% divinyl benzene as cross-linker. This support is permeable to only small molecules and ions ($\leq$100 D). The chromatographic behaviour of the tentacle supports will be fully characterised using equilibrium stirred batch kinetics (Chase, 1984) and column breakthrough analysis (Hubble, 1989). The resolving power of the most efficient tentacle support will be investigated by purification of a target protein from a crude feedstock.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 BIOCHEMICALS

Lactate dehydrogenase, lysozyme, bovine serum albumin, dextranase and dextran were all purchased from the Sigma Chemical Co., Poole, Dorset. Chicken and rabbit liver fumarase were kind gifts from the Sigma Chemical Co., Poole, Dorset. Hydroxyethyl starch was a kind gift from Oxford Nutrition Ltd., Oxford.

2.2. CHEMICALS

All reagents were analytical grade unless otherwise stated. Epichlorohydrin, iodine, terepthalaldehyde, tris(hydroxymethyl) aminoethane, potassium bromide, tetrahydrofuran, sodium chloride, allyl alcohol, methacryloyl chloride, methacrylic anhydride, zinc tetrafluoroborate, N-(hydroxy methyl) acrylamide, 2-hydroxyethyl methacrylate, sodium acetate, bromoethanol, glycidyl methacrylate, 3-allyloxy-1,2, propandiol, cyanuric chloride, divinyl sulphone, 1,4-dioxan, ammonium persulphate, pyridine, dicyclohexyl carbodiimide, sodium tetraborate, dimethylformamide, trimethyl ammonium hydroxide, o-toluidine, hydroquinone monomethyl ether packing material, molecular sieve (4 Å) and acetonitrile were all purchased from the Aldrich Chemical Co., Gillingham, Dorset.

Cibacron blue, 1,4-butanediol diglycidyl ether, sodium borohydride, L-malic acid, polyethylene imine, mercaptoethanol, Cibacron blue Sepharose CL-6B were all purchased from the Sigma Chemical Co., Poole.

Methanol, ethanol, acetone, hydrochloric acid, sulphuric acid, sodium hydroxide, sodium dihydrogen phosphate, disodium hydrogen phosphate were all purchased from FSA laboratory supplies, Loughborough, Leics.

N,N,N',N'-tetramethylethylenediamine, Coomassie Blue R-250, sodium dodecyl sulphate were all purchased from Bio-Rad Laboratories Ltd., Watford Herts.

Acrylamide (Protogel, 30% w/v) was purchased from National diagnostics, Atlanta, U.S.A

Phenol reagent (80% w/v) was purchased from Rathburn Chemicals Ltd, Scotland.

Polyvinyl alcohol (Mowiol, 98% hydrolysed, average MW 27 000) was a kind gift from Harlow Chemical Company Ltd, Harlow, Essex.

The chromatographic support (average diameter 56 μm) was a copolymer of 80% styrene and 20% divinyl benzene and was purchased from Fluka Chemicals.

2.3. EXPERIMENTAL

2.3.1. Adsorption of polyvinyl alcohol onto polystyrene beads

This method is adapted from the methods of Garvey (1974) and Tuncel *et al* (1993). Polystyrene beads (100 g, copolymer of 80% styrene and 20% divinylbenzene) were added to a stirred solution of aqueous polyvinyl alcohol (PVA, Av. Mw 27,000, 98% hydrolysed) (10 mg ml^{-1}, 1L). The PVA was allowed to adsorb for 16 h at 20°C, terephthalaldehyde (470 mg) in 100 ml dioxan was added, the suspension was then acidified with 16.7 ml of 6 M HCl and left to crosslink overnight. The beads were washed on a sintered funnel with distilled water (2 L), hot water (60°C, 1 L) methanol (200 ml) and distilled water (2 L). The support was dried on a sintered funnel and left for two days at 37°C, and stored in the dark at room temperature. The amount of PVA adsorbed to the support could be determined by analysis of the supernatant taken before and after adsorption.

2.3.2. Spectrophotometric determination of polyvinyl alcohol

This assay was adapted from the method of Zwick (1965). PVA (0.1 g) was dissolved in 10 ml of water at 80°C for 1 h. The solution was allowed to cool to room temperature and diluted 100 fold to produce a stock solution of 100 μg ml^{-1}. The stock solution was diluted serially to produce a concentration range of 5-100 μg ml^{-1}. Samples (1 ml) were placed in plastic tubes and 0.5 ml of 0.6 M boric acid and 100 μl of iodine solution ($KI:I_2$, 4:1 ratio, 50 mM I^- final conc.) was added. The samples were vortexed and left to stand at room temperature for 30 min. The absorbance was read at 690 nm against a reagent blank.

2.3.3 Determination of epoxide groups on epoxy activated PVA-polystyrene

This method has been previously described by Sundberg and Porath (1974). The extent of epoxy activation could be determined by means of a titration of immobilised epoxy groups with a pH stat (Figure 2.1). A sample of epoxy activated PVA-polystyrene support (~50 mg) was added to 1 ml of 1M sodium thiosulphate pH 7. The release of OH^- was followed by titration with 10 mM HCl to maintain the neutral pH. The volume of acid added was used to calculate the number of immobilised epoxy groups.

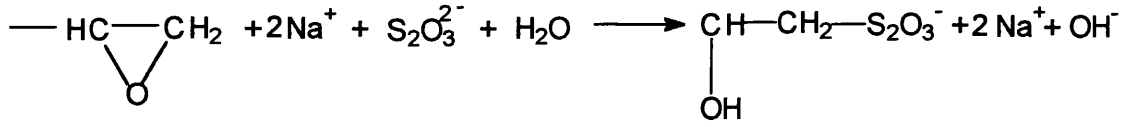


Figure 2.1: *Reaction of epoxy groups with sodium thiosulphate*

2.3.4. Phenol-sulphuric acid determination of polysaccharides

The assay was is based on the method of Dubois (1965). A standard curve was prepared by dilution of a stock polysaccharide solution (100 μg ml^{-1}) to produce a concentration range between 5-80μg ml^{-1}. Phenol reagent (50 μl, 80 % (w/v)) was added to 2 ml of sample and 5 ml of concentrated sulphuric acid was rapidly added. The samples were allowed to cool for 10 min, vortexed and placed in a water bath at 25°C for 15 min. The absorbance at 690 nm was determined against a reagent blank.

2.3.5. Determination of adsorbent volume

A settled volume of gel (1 ml) was dried on a glass sinter for 5 minutes. It was then transferred to a pre-weighed bottle and dried at 37°C for two days. The mass of dry support was equivalent to 1 ml of adsorbent. This value was used in the determination of adsorbent volume used in all protein binding experiments.

2.3.6. Drying of organic solvents

Anhydrous solvents were purified by the methods of Perrin and Armarego (1990). Molecular sieve (4 Å) was activated by heating in an oven at 300°C overnight and left in an oven at 80°C prior to use.

2.3.6.a. Acetonitrile, pyridine, and triethylamine

The solvent (1 L) was placed in a round bottom flask and 2g of calcium hydride was added the suspension was stirred for one hour and then was refluxed for two hours. The solvent was distilled and stored over molecular sieve (4 Å).

2.3.6.b. Methanol

Magnesium turnings (5g) and 0.5 g of iodine were added to methanol (60 ml). The suspension was warmed (40° C) until the iodine disappeared and all the magnesium was converted to methoxide. Methanol (800 ml) was added and after refluxing for 3 hours, was distilled and stored over molecular sieve (4 Å).

2.3.7. Determination of protein concentration

Protein was determined using the Bio-Rad assay reagent (diluted 5 fold, 800 µl) was added to appropriate serial dilution's of the protein solution (200 µl) and vortexed. After 5 minutes at room temperature the absorbance was measured at 595 nm against a reagent blank, bovine serum albumin was used for the protein standard curve.

2.3.8. Polyacrylamide gel electrophoresis

Electrophoresis was carried out using the discontinuous buffer system of Laemmli (1970) and the method described by Hashimoto *et al.,* (1983). The linear slab gels were 10% acrylamide. Stacking gel (acrylamide 6.375% (w/v), 0.156 M Tris-HCl, 0.125% SDS, pH 6.8) and resolving gel (acrylamide 10% (w/v), 0.5 M Tris-HCl, 0.135% SDS, pH 8.8) were used to electrophorese the loaded samples (25 µl). All samples were boiled in an equal volume of sample buffer; (40 mM Tris-HCl (pH 6.8), 20% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 0.00025% (w/v) bromophenol blue). The gels were run at 200V for one hour before staining with 0.1% (w/v) Coomassie blue

R-250 in 40% (v/v) methanol 10% (v/v) acetic acid for one hour. The gel was destained overnight with 40% (v/v) methanol 10% (v/v) acetic acid.

2.3.9. Stop flow experiments

Stop flow experiments were carried out using a Hi-Tech Scientific SF 61-MX multimixing stop flow system which was linked to a computer for data analysis. Two reservoirs are filled, one with enzyme and the other with a dye a-polymer conjugate. Two syringes are charged directly from the reservoirs. Temperature was controlled via a thermostated water bath. When the trigger was actuated, a piston drives the contents of the syringes through a mixing chamber and into a flow cell before reaching the stop syringe which brings the system to a halt. The volume of reactant discharged from each syringe was 75 µl and the concentration of reactants was twice that of the final concentration in the mixing cell. The system was triggered twice to ensure that reactants had removed any previous contaminants from the system before kinetic data was collected. Software supplied with the machine was used to analyse and fit the raw data to suitable models.

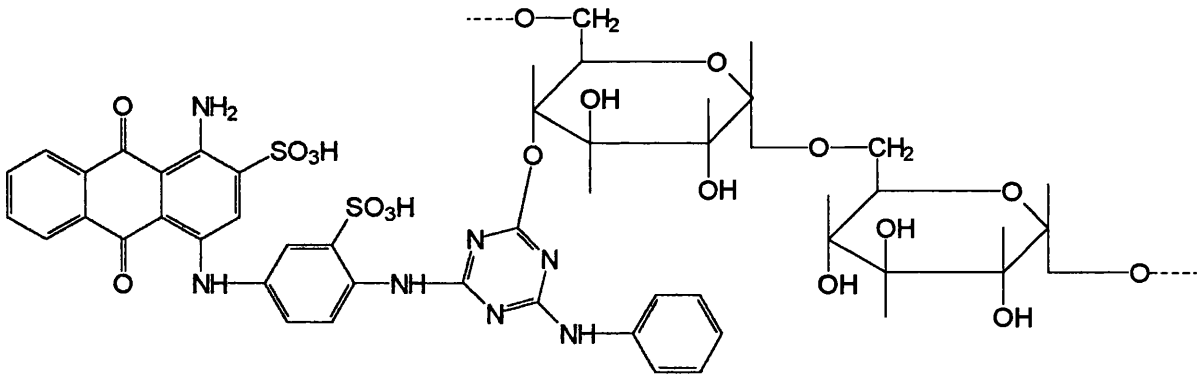
CHAPTER THREE

IMMOBILISATION OF DYE-POLYMER CONJUGATES

3.1. INTRODUCTION

One of the first tentacle supports was used by Thompson and co-workers (1975), blue dextran (Mw 2000) was immobilised on Sepharose. The small polymer size enabled protein diffusion to occur into the porous matrix, thus protein binding was not restricted to the external surface.

One of the project aims was to immobilise pre-characterised soluble polymers and compare equilibrium and kinetic data after immobilisation. A pre-characterised polymer such as blue dextran (figure 3.1) which had been fully characterised by Mayes (1992) and co workers.



Blue dextran

Figure 3.1: *The structure of the blue dextran conjugate*

Who found that;

- regardless of dye loading a constant 28% of the total coupled dye molecules was available for high affinity protein binding at saturation;
- The dissociation constant (K_d) for the dye-protein interaction decreased as the dye loading on the dextran increased;
- There was evidence of a high affinity binding site and a low affinity binding site.

Dextran is a storage polymer synthesised by the organism *L. mesenteroides*. Dextran fits many of the criteria needed for a tentacle polymer: readily derivitisable, highly water soluble, chemically stable and a large number of groups to enable attachment to a chemically modified support.

Dextran is one of the components used along with polyethylene glycol to make two immiscible phases for aqueous two phase protein separations (Kopperschlager and Birkenmeier, 1986). Thus blue dextran was logical choice to be used as a tentacle polymer.

An alternative to dextran is to use a natural polymer which has been modified with primary hydroxyls. This may increase the amount of polymer immobilised on to the support. An example is hydroxyethyl starch (HE-starch) which resembles amylopectin and consists of 1,4-α glycosidic bonds (figure 3.2). There are 6.2 hydroxyethyl groups per 10 glucose residues, and the polymer is hydroxylated in the C-2 position.

Dextran and HE-starch are natural polymers; they are composed of glucose molecules which makes them susceptible to biological attack from micro-organisms. Thus synthetic polymers such as polyvinyl alcohol and polyethylene imine were also considered. These two polymers have the advantage of being linear with a polymethylene back bone which is resistant to biological attack. Polyvinyl alcohol is a neutral polymer (figure 3.2) and is ideal for use as a tentacle polymer. Polyethylene imine is not neutral (figure 3.2) as it consists of primary, secondary and tertiary amine groups (Geckeler *et al.*, 1980).

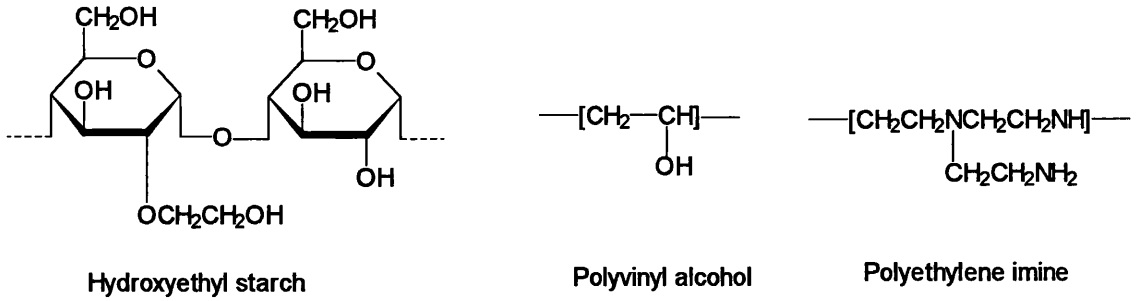


***Figure 3.2:** Structures of hydroxyethyl starch, polyvinyl alcohol and polyethylene imine*

Thus any groups which are underivitised will be charged. This ion exchange effect may hamper or improve protein binding and will be investigated.

Polyethylene imine has been used as an ion exchange tentacle polymer (Kitagawa, 1987) so it will be interesting to see how the polymer compares when an affinity ligand is coupled to the polymer.

3.2. DEXTRAN

3.2.1. Coupling of Cibacron blue to dextran

This method has been previously described by Mayes *et al* (1992). Dextran (20 g, average Mw 0.5 x10^6) was dissolved in 1L of 188 mM Na_2CO_3 and 1 g of Cibacron blue was added. The mixture was incubated at 45°C for 24 hours. A 100 ml aliquot was removed and more dye (1g) was added to achieve the next highest loading. This cycle was repeated a further 8 times to give a range of dye-conjugates. An equal volume of ethanol was added to each sample together with a few crystals (~ 50 mg) of sodium acetate trihydrate, and left overnight at -20°C. The precipitate was centrifuged at (27, 000g) for one hour at 4°C and the pellet was resuspended in 50 ml of water. The precipitation and resuspension was repeated until the supernatant had an absorbance of less than 0.01 at 595 nm. The solutions were dialysed against 10 L of water. The volume was reduced to about a third of the original volume (~ 100 ml) using a rotary evaporator. A sample (4 ml) was taken to determine the dye loading. The remainder was frozen in a round bottom flask using liquid nitrogen and freeze dried.

3.2.2. Determination of dextran dye loading

a) Dry weight determination

Samples (1 ml) of the blue dextran conjugates were pipetted in triplicate into preweighed glass tubes and were dried in an oven at 80°C to constant weight. All the tubes were weighed and the mass of conjugate in each tube calculated. These values were combined with spectrophotometric measurements of the same conjugates in order to calculate the dye loading.

b) Hydrolysis of dye dextran conjugates

This method was based on the method described by Chambers (1977). The oven dried conjugates (section 3.2.2a) were dissolved by incubation with 1 ml of 6 M HCl at 45°C for 10 minutes. The resulting purple solutions were diluted if necessary with 6 M HCl and the absorbance at 541 nm was measured using 6 M HCl as a blank. The dye concentration was calculated using an extinction coefficient of 3985 M^{-1} cm^{-1} (Mayes 1990).

3.2.3. Epoxy activation of PVA-polystyrene

This method has been previously described by Turkova *et al* (1981). PVA-polystyrene beads (5 g) were placed in 50 ml of 10 M KOH for half an hour and were dried on a sintered funnel. The beads were refluxed in 20 ml of epichlorohydrin for one hour. 3.5 ml of saturated KBr was added and the suspension was refluxed for 3 hours. The beads were then washed with acetone (50 ml), acetone/water (50 % v/v, 50 ml) and water (200 ml). The beads were refluxed in 25 ml of zinc tetrafluoroborate (40% w/v in water) for 3 hours the beads were washed with 1 L of water.

The beads were placed in 7.5 ml of 0.6 M NaOH which contained sodium borohydride (2 mg ml^{-1}). An equal volume of 1,4-butanediol diglycidal ether was added dropwise and the suspension was left stirring overnight at room temperature (Sundberg and Porath, 1974).

3.2.4. Coupling of blue dextran to epoxy PVA-polystyrene

Epoxy PVA-polystyrene (10 g) was added to 10 ml of 0.25 M NaOH which contained blue dextran at 30 mg ml^{-1} and was left at 45°C overnight. The beads were washed with copious amounts of water until the washings were no longer blue. The beads were packed into a column and washed with; 1 M NaCl (200 ml), 50 % (v/v) ethanol/water (200 ml), 1 M NaCl (100 ml) and finally with buffer (10 mM Tris 50 mM NaCl pH 8.0). The beads were stored in this buffer at 4°C with a few crystals of sodium azide.

3.2.5. Immobilisation of blue dextran via primary hydroxylation of PVA-polystyrene

PVA-polystyrene (2g) was stirred in 20 ml of 1M NaOH at 60°C and 2 ml of (N-hydroxy methyl) acrylamide (0.5 M) was added and the mixture was stirred for two hours (Pittfield, 1994). The beads were washed with water and were added to 2.5 ml of 2 M NaOH which contained sodium borohydride (2 mg ml^{-1}). Epichlorohydrin (1.25 ml) was added dropwise and the suspension was left stirring overnight at room temperature.

The beads were washed with water (500 ml), acetone (50 ml) and water (250 ml), the beads were added to blue dextran (30 mg ml^{-1}) in 10 ml of 0.25 M NaOH 20% (v/v) dimethyl sulphoxide (DMSO) and left at 45°C for 4 hours. The beads were washed with water, packed into a small column and washed with 200 ml each of ethanol/water (50% v/v), 1M NaCl and water. The beads were stored in water at 4°C with a few crystals of azide.

3.2.6. Divinyl sulphone activation and coupling of blue dextran to PVA-polystyrene

This activation method was described by Sundberg and Porath (1974). PVA-polystyrene (4 g) was stirred in 2 M Na_2CO_3 (2 ml, pH 11); 0.4 ml of divinyl sulphone was added slowly over 15 min and the suspension was left for one hour. The activated beads were washed with water (200 ml) and placed in blue dextran (20% w/v, in 2 M Na_2CO_3, 6 ml) overnight at room temperature. The beads were washed with water, packed into a small column and washed with 200 ml each of ethanol/water (50% v/v), 1M NaCl and water. The beads were stored in water at 4°C with a few crystals of azide.

3.2.7. Cyanuric chloride activation and coupling of blue dextran to PVA-polystyrene

This method was developed by Pittfield, (1993). PVA-polystyrene (5 g) was placed in 50 ml of 1 M NaOH and was dried on a glass sinter. The beads were added to a solution of cyanuric chloride (100 mM, 50 ml) in dry acetone for 10 minutes, and washed with 80 ml each of; acetone, 50% (v/v) acetone/water and water. The support was placed in coupling buffer (50 ml) which contained blue dextran (4% (w/v)) in 0.5 M Na_2CO_3, 1 M NaCl pH 12.5. The beads were stirred at 60°C for 4 hours and washed on a sinter with water, packed into a small column and washed with 300 ml each of 50% (v/v) ethanol/water, 1M NaCl and water. The support was stored at 4°C with a few crystals of azide.

3.2.8. Dye loading determination of immobilised blue dextran polystyrene

Oven-dried blue dextran polystyrene (800 mg) was incubated with 1.2 ml of 6 M HCl at 45°C. The suspension was vortexed and placed in an eppendorf tube which had a 0.45 µm polypropylene filter and spun at 3000 g for 3 minutes. The absorbance of the supernatant was measured against 6 M HCl as a blank. An extinction coefficient of 3985 M^{1} cm^{-1} (Mayes 1990) was used to determine the dye concentration.

3.2.9. Frontal analysis of lysozyme binding to immobilised blue dextran polystyrene

Immobilised blue dextran polystyrene (1 ml, section 3.2.4.) was packed into a column and equilibrated with degassed buffer (10 mM Tris 50 mM NaCl pH 8.0) at a flow rate of 66.7 µlmin^{-1} for 2 hours. Lysozyme at a concentration of 84 µgml^{-1} in the same buffer was loaded continuously onto the column at the same flowrate. The column effluent was pumped through a spectrophotometer equipped with a UV flow cell and the trace was recorded with a chart recorder. The experiment was stopped when the inlet concentration matched the effluent concentration. A frontal analysis model (Hubble, 1989) was used to fit the trace obtained.

3.3. HYDROXYETHYL STARCH

Hydroxyethyl starch (HE-Starch, 500 ml) which was supplied as a 6% (w/v) solution in sterile saline, was divided into six equal portions and two equivalent volumes of ethanol were added along with a few crystals of sodium acetate trihydrate and left at -20°C overnight. The polymer was pelleted by centrifugation at 27 000g at 4°C. The pellets were resuspended in a minimum volume of water and pooled. The polymer was dialysed against water (5L) overnight at 4°C and freeze dried.

3.3.1. Coupling of Cibacron blue to HE-Starch

HE-starch (4.5 g) was dissolved in 200 ml of 188 mM Na_2CO_3 and 200 mg of Cibacron blue was added and the suspension was incubated at 45°C overnight. Dye (200 mg) was added daily (5 additions). The polymer was precipitated with two equal volumes of ethanol and a few crystals of sodium acetate trihydrate and left at -20°C overnight. The precipitate was centrifuged at 27 000 g at 4°C and the pellet was resuspended in 200 ml

of 188 mM Na_2CO_3. A further five additions of dye were made. The dye-polymer was precipitated and excess dye was removed as described in section 3.2.1.

3.3.2. Dye loading determination of blue HE-Starch

The determination of dye loading was carried out as described in section 3.2.2a and 3.2.2.b.

3.3.3. Immobilisation of blue HE-starch to PVA-Polystyrene

PVA-polystyrene (10 g) was placed in 100 ml of 1 M NaOH and 10 ml of (N-hydroxymethyl) acrylamide (0.5 M) was added. The suspension was stirred at 60°C for 2 hours. The beads were washed with 200 ml of water and placed in 17 ml of 2 M NaOH which contained sodium borohydride at 2 mg ml^{-1}, 8.5 ml of epichlorohydrin was added dropwise and the suspension was stirred overnight. The epoxy activated beads were placed in 30 ml of 0.25 M NaOH which contained blue HE-starch (250 mg ml^{-1}). The suspension was stirred at 50°C for 5 hours. The beads were washed on a sintered funnel with water until the filtrate was clear. The beads were packed into a 25 ml column and washed sequentially with 400 ml of 50 % (v/v) ethanol/water and 250 ml of 1 M NaCl, 150 ml of water and finally 50 M sodium phosphate pH 7.9. The beads were stored in sodium phosphate buffer with a few crystals of sodium azide at 4°C.

3.3.4. Dye loading determination of immobilised blue HE-starch

The amount of immobilised dye and hence the amount of immobilised polymer was determined as described in section 3.2.8.

3.3.5. Spectral titrations of blue HE-starch

Sample and reference cuvettes each containing a solution of 50 μM (with respect to dye) conjugate in 50 mM sodium phosphate pH 7.9 were placed in a Cecil 6000 spectrophotometer. Small volumes of 0.25 mM (with respect to subunits) lactate dehydrogenase or 5 mM lysozyme was added to the sample cuvette, and equal volumes of buffer were added to the reference cuvette. The cuvette contents were mixed with a small paddle which was placed in situ without blocking the light path. The change in absorbance was measured after each addition of lactate dehydrogenase by scanning from 500-700 nm. The titration was stopped when there was no further increase in the

magnitude of the difference spectrum. Lactate dehydrogenase was used in the concentration range 0-50 μM (with respect to subunits). Lysozyme was used in the concentration range 0-300 μM.

3.3.6. Stop flow experiments

The experiments were carried out using stop flow apparatus (section 2.3.9). Syringes were filled with 240 μM (with respect to subunits) lactate dehydrogenase in 100 mM sodium phosphate buffer pH 7.9 and 100 μM blue HE-starch conjugate (with respect to dye). The data was collected on computer and fitted with a single exponential model. The experiment was repeated as described previously, except that the LDH concentration was 120 μM (with respect to subunits).

3.3.7. Equilibrium stirred batch kinetic experiments

This apparatus was previously described by Horstmann and Chase (1986). A water jacketed vessel maintained at 25°C was set up with an overhead stirrer and a 20 μm porosity filter. The filter led to an external loop which contained a Cecil 272 spectrophotometer equipped with a UV flow cell and a peristaltic pump. Buffer (10 mM Tris 50 mM NaCl pH 8, 50 ml) which contained lysozyme (concentration range 15-500 $\mu g ml^{-1}$) was pumped around the system at a fast flowrate (2 ml min^{-1}) to reduce the response time. At time zero 1.4 g (~2 ml) of sintered dried adsorbent was added to the vessel and the trace was recorded with a computer data compiler. The amount of protein bound could be determined by the difference between the two plateaus, or by using the model of Chase (1984).

3.4. POLYETHYLENE IMINE

3.4.1. Synthesis of blue polyethylene imine

Cibacron blue was coupled to polyethylene imine (PEI) using the same procedure for HE-starch (section 3.3.1) with the exception that, 20 g of PEI was dissolved in 1L of 188 mM Na_2CO_3 and 1 g of dye was added. The addition cycle was repeated 10 times. The polymer was precipitated and resuspended in 1 L of Na_2CO_3 followed by a further 10 additions of dye.

3.4.2. Determination of extinction coefficient of Cibacron blue in DMF (50% v/v)

Deviation from the Beer-Lambert law was investigated to determine if the extinction coefficient of Cibacron blue in DMF/water (50% v/v) has the same value as the extinction coefficient of the dye in water. DMF/water (50% (v/v), 2 ml) was placed in a quartz cuvette and placed in a dual beam Cecil 660 spectrophotometer. Additions of a concentrated stock solution of dye in 50% (v/v) DMF/water were made to the cuvette using a positive displacement pipette. The contents were mixed with a paddle in the cuvette. The absorbance at 610 nm was recorded. Further additions of dye were made until the absorbance reached a value of 1.

3.4.3. Dye loading determination of blue-PEI

A sample (15 mg) of the freeze dried blue-PEI was dissolved in 2 ml of 50% (v/v) DMF/water, the resulting solution was diluted if necessary with 50% (v/v) DMF/water. The absorbance at 610 nm was measured. The dye loading could then be determined.

3.4.4. Immobilisation of blue-PEI to epoxy PVA-polystyrene

PVA-polystyrene (20 g) was suspended in 200 ml of 2M NaOH at 60°C, and hydroxyethyl methacrylate (20 ml) was added to the suspension and stirred for two hours. The beads were epoxy activated (as described in section 3.3.3), using 38 ml of 2 M NaOH which contained sodium borohydride at 2 mg ml^{-1} and 19 ml of epichlorohydrin.

Blue-PEI (1 g) was dissolved in 20 ml of 50% v/v DMF/water at 60°C and centrifuged (5000 g) to remove undissolved blue-PEI. The blue PEI solution was added to trimethyl ammonium hydroxide (6.25 g). Epoxy PVA-polystyrene (20g) was added to the coupling solution and left for 24 hours at 60°C. The beads were washed on a sintered funnel with water until the filtrate was clear. The beads were then packed into a column and washed sequentially with; 400 ml of 50 % (v/v) ethanol/water, 250 ml of 1 M NaCl, 150 ml of water and finally 100 ml of 50 mM sodium phosphate pH 7.9. The beads were stored in sodium phosphate buffer with a few crystals of sodium azide at 4°C.

3.4.5. Dye loading determination of immobilised blue-PEI

Oven dried blue-PEI polystyrene (800 mg) was incubated with 1 ml of 6 M HCl at 45°C. The suspension was placed in a small round bottom flask and freeze dried. 2 ml of 50% (v/v) DMF/water was added to the flask. The suspension was then vortexed and placed in eppendorf tubes which had a 0.45 μm polypropylene filter and spun at 3000 g for 3 minutes. The absorbance of the supernatant was measured against 50% (v/v) DMF/water as a blank.

3.4.6. Equilibrium stirred batch kinetic experiments

Experiments were carried out as described in section 3.3.7, except that the experiments were carried out at different pH. The following buffers (50 mM) were used sodium acetate pH 5, potassium phosphate pH 6 and 7, sodium phosphate pH 7.9 and sodium borate pH 9.

3.5. POLYVINYL ALCOHOL

3.5.1. Synthesis of hydroxyethyl polyvinyl alcohol

This method was based on that of Chun *et al* (1990). Polyvinyl alcohol (10g, MW 27,000) was dissolved in 100 ml of 1.5 M NaOH at 100°C and then stirred at 80°C for 24 hours to introduce alkoxide groups into the polymer. Bromoethanol (20 ml) was added to the solution. A condenser was placed onto the round bottomed flask and stirred at 80°C for 16 hours. The resulting amber coloured solution was neutralised with 10 M NaOH and the polymer was precipitated by addition of acetone (~2L). The polymer was centrifuged and the pellets were resuspended in water (50°C), pooled and then dialysed against water (10 L) and freeze dried.

3.5.2. Coupling of Cibacron blue to hydroxyethyl polyvinyl alcohol

The primary hydroxylated PVA polymer (4 g) was dissolved in 200 ml of 2M Na_2CO_3; 200 mg of Cibacron blue was added and the mixture was incubated at 45°C for 24 hours. The cycle was repeated four times with 200 mg of dye added at each stage. The polymer was precipitated by the addition of three equal volumes of ethanol and a few crystals of sodium acetate trihydrate and allowed to stand at -20°C overnight. The precipitate was

spun down (27 000g) at 4°C, the pellet was resuspended in 50 ml of water. The precipitation and resuspension were repeated until the supernatant had an absorbance of less than 0.01 at 595 nm. The pellet was finally resuspended in 50 ml of water and dialysed against a large excess of distilled water (5 L). The polymer was then freeze dried.

3.5.3. Dye loading determination of blue-hydroxyethyl polyvinyl alcohol

The determination of dye loading was carried out as described in section 3.2.2a and 3.2.2b.

3.5.4. Coupling of blue-hydroxyethyl polyvinyl alcohol to epoxy PVA-polystyrene

Coupling of blue-hydroxyethyl polyvinyl alcohol to epoxy activated PVA-polystyrene was carried out as described in section 3.3.3.

3.5.5. Dye loading determination of immobilised blue-hydroxyethyl polyvinyl alcohol

The amount of immobilised dye and hence the amount of immobilised polymer was determined as described in section 3.2.8.

3.5.6. Equilibrium stirred batch kinetics experiments using immobilised blue-hydroxyethyl polyvinyl alcohol

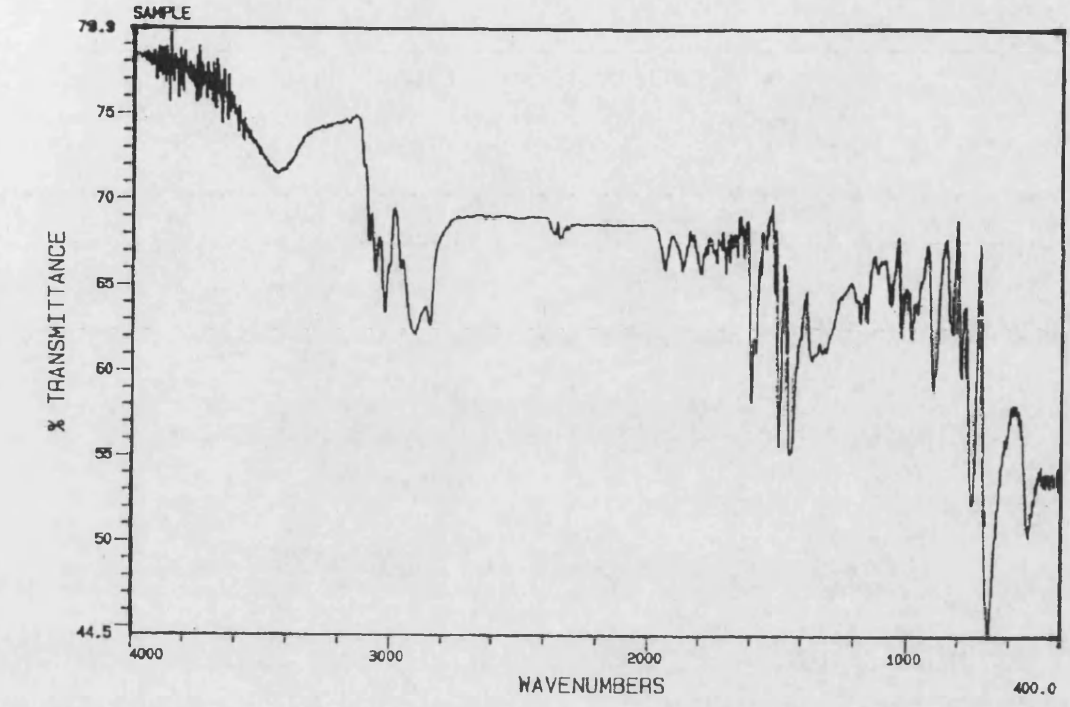
The experiments were carried out as described in section 3.3.7.

3.6. RESULTS and DISCUSSION

3.7. BLUE DEXTRAN

3.7.1. Adsorption of PVA onto polystyrene

The polystyrene support (copolymer of 80% styrene and 20% divinyl benzene) was chosen because the beads are non-porous. A bead manufactured by Biorad, which contains 12% divinyl benzene had an exclusion limit of 400 Daltons. Thus a polystyrene bead with 20% divinyl benzene would be permeable only to very small molecules. PVA was adsorbed quite easily to the polystyrene surface. This gives polystyrene a hydrophilic coating and masks the hydrophobic surface. This was evident by the observation that the PVA-polystyrene beads settled in the aqueous reaction medium. It is likely that PVA is adsorbed to the surface of polystyrene with hydrophobic interactions, London and Van der Waals forces. Figure 3.3 shows an IR spectrum of native polystyrene and PVA coated polystyrene. A larger OH stretch can be seen at about 3400 cm^{-1} for the PVA-polystyrene. The hydrophilic coating was made permanent by cross linking with terepthaladehyde (Tuncel *et al* 1992) which produced beads which sediment easily.



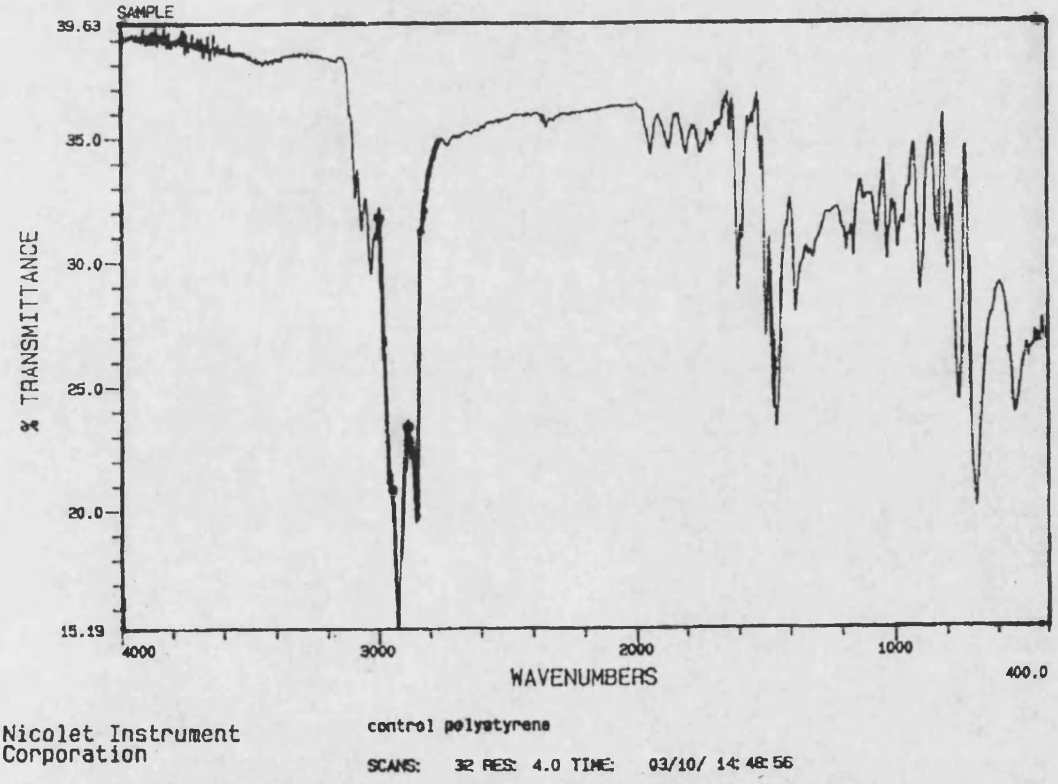


Figure 3.3: IR spectra of PVA-polystyrene and native polystyrene beads
Samples of the supports (~5mg) were mixed with KBR and pressurised to form discs. A blank KBr disc was made and the samples were analysed with a IR Nicolet spectrophotometer.

3.7.2. Coupling of blue dextran conjugates to epoxy PVA- polystyrene

PVA-polystyrene beads could not be activated using the usual conditions for agarose which can react readily with either epichlorohydrin (Porath and Olin 1983) or bisepoxide (Porath *et al.*, 1975). This is because the secondary hydroxyls of the PVA are molecule are not particularly reactive. Thus reaction conditions to introduce epoxide groups were quite harsh, however the method worked well and demonstrated that the beads are able to withstand 10 M KOH (section 3.2.3.) without obvious damage. Attempts to couple blue dextran conjugates directly to the epoxy activated beads failed. A catalyst (zinc tetrafluroborate) was therefore used to open the epoxy groups. The resulting support could readily react with bisepoxide.

This allowed blue dextran conjugates to be immobilised on to the beads under alkaline conditions. Two dye conjugates were immobilised. Figure 3.4 shows the reaction scheme. Table 3.1 gives details of the immobilised blue dextran polystyrene supports.

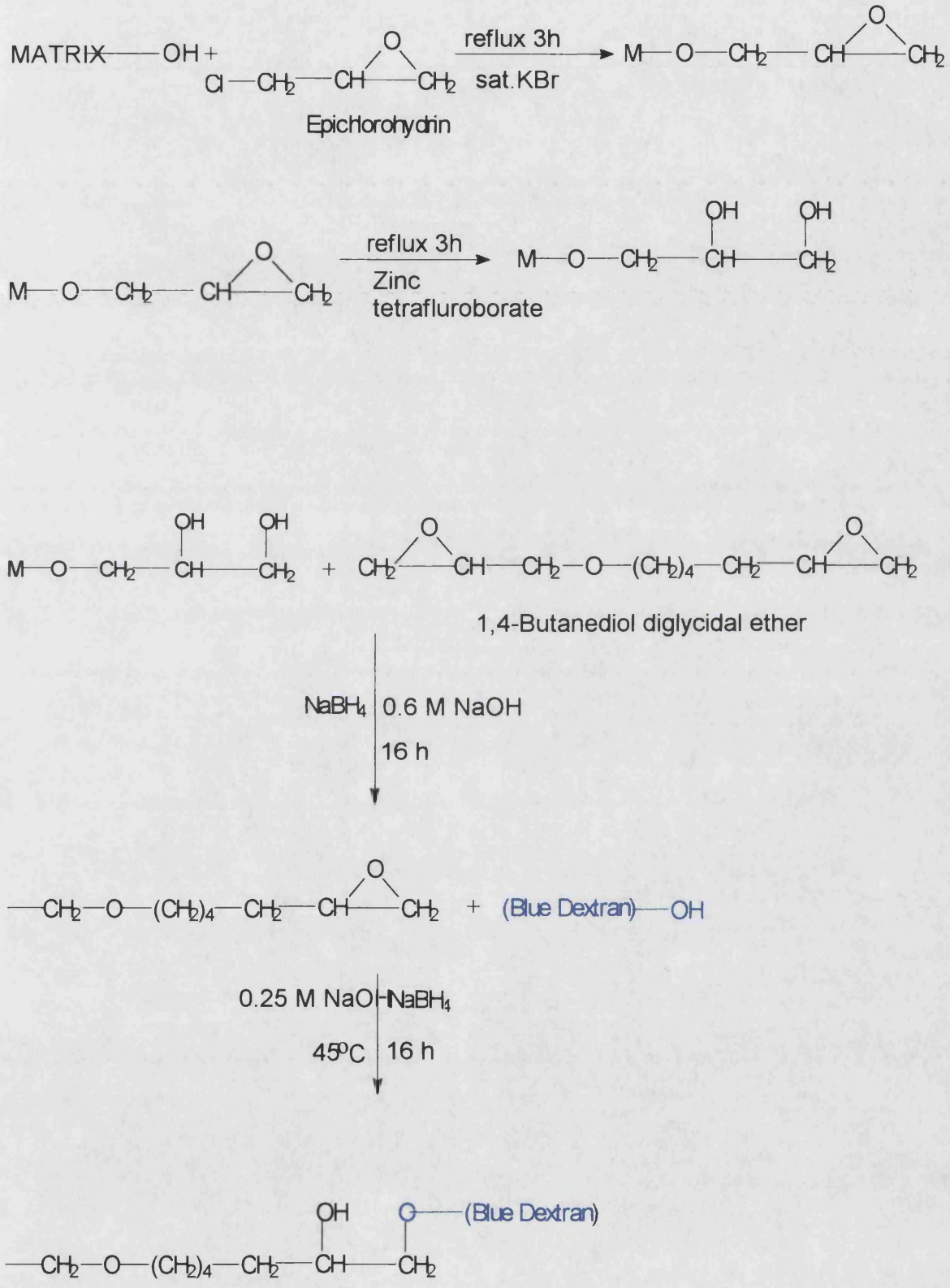


Figure 3.4: *Reaction scheme for the synthesis of immobilised blue dextran onto PVA-polystyrene*

Table 3.1. Results for the synthesis of immobilised blue dextran polystyrene

Dye-dextran code	Dye-dextran loading moles of dye/moles of dextran	Amount of dextran bound ($\mu g\ ml^{-1}$ adsorbent)	Amount of dye bound (nmole ml^{-1} adsorbent)
5D5	58	400	54
5D9	145	150	52

The immobilised blue dextran polystyrene beads were a very pale blue. This was due to the small amount of blue dextran conjugate immobilised (52 and 54 nmole ml^{-1} respectively). In comparison blue Sepharose CL-6B has a dye ligand density of 4 μmole ml^{-1}. Interestingly, there is approximately 2.5 times as much lower loaded dextran (5D5) bound, compared with the higher loaded dextran (5D9). Mayes (1992) showed that the more highly substituted dextrans had a smaller hydrodynamic radius and speculated that this was due to greater hydrophobic interactions between dye molecules, causing the molecule to compact. This reduces the number of free hydroxyls available to interact with the epoxide groups on the bead surface.

The coupling experiment was repeated with higher conjugate concentrations but the amount of blue dextran bound could not be improved. Alternative activation procedures were attempted using divinyl sulphone (Porath, 1974) and cyanuric chloride (Biagioni *et al.*, 1978; Pittfield, 1994). These activation procedures also produced supports with low amounts of immobilised blue dextran.

Non-aqueous solvents were used in an attempt to reduce the amount of dye-dye interactions, which may cause a reduction of hydroxyl groups to bind the bead surface. Of the solvents tested; DMF, dioxan, THF and acetonitrile precipitated the dye-polymer. Only DMSO/water (20% v/v) was suitable. However, the presence of DMSO did not increase the amount of immobilised blue dextran.

3.7.3. Column breakthrough analysis

Blue dextran polystyrene was packed into a small column and was subjected to a constant flow of lysozyme solution. The experiment was terminated when the effluent concentration matched the inlet concentration. The results are shown in figures 3.5 and 3.6. The traces were fitted to a model (Hubble, 1989) which predicts when saturation of the column occurs. Although the sigmoid shape of the elution curve is similar to that predicted by the model, there is a systematic deviation of the experimental data from the fitted curve. This can be explained by the fact that the model describes the behaviour for porous beads such as agarose, whilst the blue dextran polystyrene is a non-porous bead. A non-porous bead would behave in a different manner especially due to very low diffusion limitations.

A)

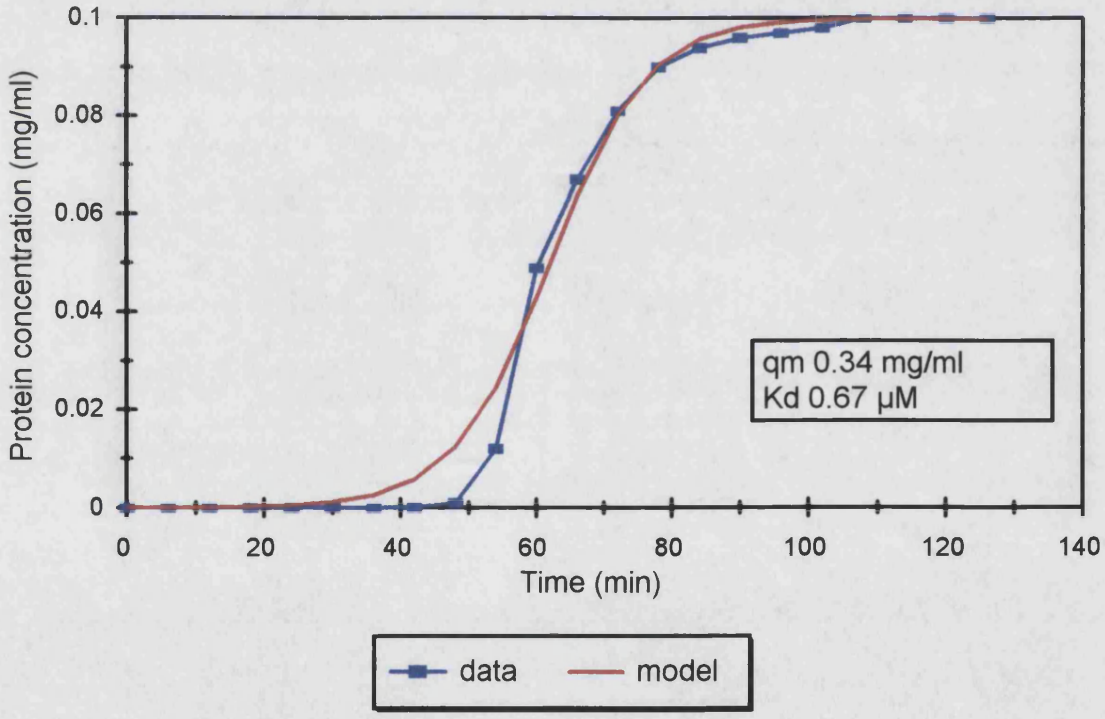


Figure 3.5: *Frontal analysis of lysozyme binding to immobilised blue dextran conjugate (5D9).*

Immobilised blue dextran polystyrene (1 ml) was packed into a column and equilibrated with degassed buffer (10 mM Tris 50 mM NaCl pH 8.0) at a flow rate of 66.7 µl min^{-1} for two hours Lysozyme at a concentration of 45 $\mu g ml^{-1}$ in the same buffer was loaded continuously onto the column at the same flowrate. The column effluent was pumped through a spectrophotometer equipped with a UV flow cell and the trace was recorded with a chart recorder, the experiment was stopped when the inlet concentration matched the effluent concentration. A frontal analysis computer model (Hubble, 1989) was used to fit the trace obtained.

B)

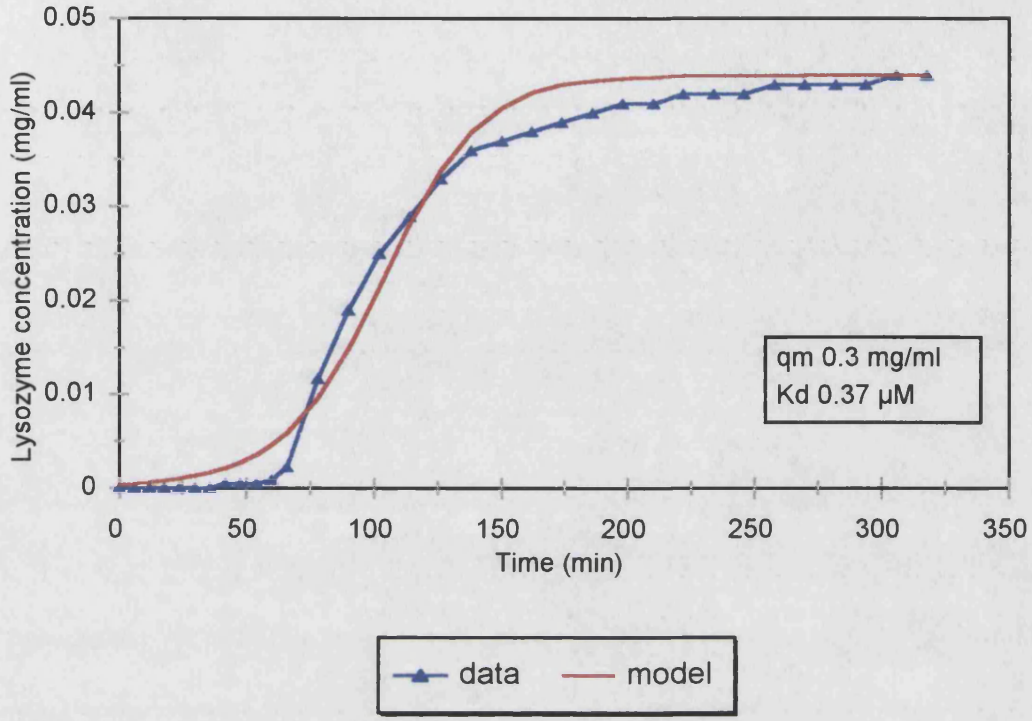


Figure 3.6: *Frontal analysis of lysozyme binding to immobilised blue dextran conjugate (5D5).*

Immobilised blue dextran polystyrene (1 ml) was packed into a column and equilibrated with degassed buffer (10 mM Tris 50 mM NaCl pH 8.0) at a flow rate of 66.7 µl min^{-1} for two hours Lysozyme at a concentration of 100 µgml^{-1} in the same buffer was loaded continuously onto the column at the same flowrate. The column effluent was pumped through a spectrophotometer equipped with a UV flow cell and the trace was recorded with a chart recorder, the experiment was stopped when the inlet concentration matched the effluent concentration.. A frontal analysis computer model (Hubble, 1989), was used to fit the trace obtained.

Estimates of the binding capacity (q_m) of both immobilised blue dextran polystyrene supports were very low 0.3 mg ml^{-1} for 5D5 blue dextran conjugate and 0.34 mg ml^{-1} for the 5D9 blue dextran conjugate. In comparison blue Sepharose has a binding capacity of 16 mg ml^{-1} for lysozyme (Horstmann *et al.*, 1986). The estimates of the dissociation constant (K_d) were of the order of 10^{-7} M, which shows the supports have a high affinity for lysozyme. Blue Sepharose has a dissociation constant for lysozyme of 4.86 x 10^{-6} M (Horstmann *et al*, 1986). The low capacity and low dye loading on the blue dextran polystyrene supports meant that very little could be achieved with such supports.

3.8. BLUE HYDROXYETHYL-STARCH

3.8.1. Synthesis of HE-starch

Hydroxyethyl starch (HE-starch) was substituted with Cibacron blue. The conjugate had a dye loading of 47 moles of dye: mol dextran, assuming a molecular weight of 200 kD (Oxford Nutrition) for the HE-starch. This dye-conjugate was chosen for immobilisation because of the high dye loading (equivalent to 117 mol dye:mol dextran if based on a polymer of 0.5×10^6 Da). This is similar to the dye loading of the blue dextran conjugate that was immobilised (5D9, 145 mol dye:mol dextran).

3.8.2. Spectral titrations and stop flow experiments of blue HE-starch

Spectral titrations were carried out using blue HE-starch which was titrated with lysozyme. A typical spectrum is shown in figure 3.7. At higher concentrations of lysozyme the initial absorbance began to increase. This effect caused some of the troughs at 595 nm to be displaced upwards. This was due to precipitation in the cuvette. This phenomenon was also observed for blue dextran by Mayes (1992). Data from these difference spectra are interpreted by measuring the amplitude of the trough at 595 nm (Mayes 1992). The magnitude of the absorbance change is directly proportional to the concentration of the lysozyme-dye complex, which gives a quantitative estimate of the complex concentration.

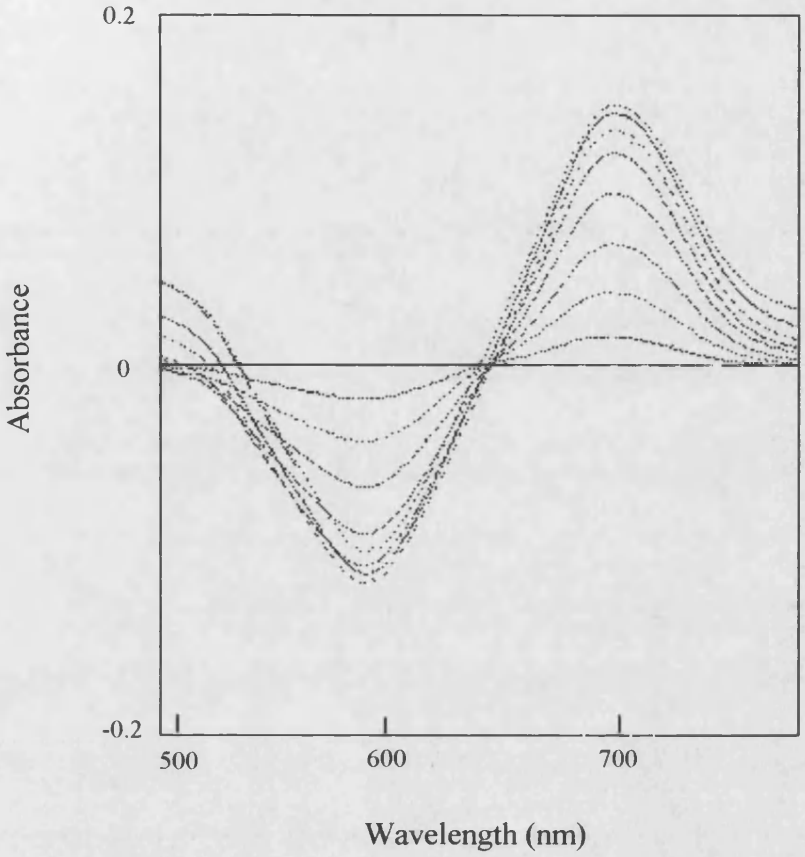


Figure 3.7: *Spectral titration of blue HE-starch with lysozyme*
Sample and reference cuvettes each containing a solution of 50 μM (with respect to dye) conjugate in 50 mM sodium phosphate pH 7.9, were placed in a Cecil 6000 spectrophotometer. Small volumes of 5 mM lysozyme was added to the sample cuvette, and equal volumes of buffer were added to the reference cuvette. The cuvette contents were mixed with a small paddle which was placed in situ, without blocking the light path. The change in absorbance was measured after each lysozyme addition, by scanning from 500-700 nm. The titration was stopped when there was no further increase in the magnitude of the difference spectrum. Lysozyme was used in the concentration range 0-300 μM.

The interaction can be modelled by using a simple mutual depletion model. The model describes the equilibrium between bound dye and free dye in solution by taking account the difference between the spectra for free and bound dye. The overall absorbance of the system can be described as follows;

$$A_{595} = \varepsilon_f [D_f] + \varepsilon_b [D_b] \qquad (3.1)$$

Where ε_f and ε_b are the extinction coefficients for the free and bound dye respectively, at the chosen wavelength and D_f and D_b are their concentrations. When measuring the difference spectra between two solutions of equal dye concentration with only one

containing protein, then the change in absorbance at 595 nm between the two solutions is given by;

$$\Delta A_{595} = [D_b]\,(\varepsilon_b - \varepsilon_f) \qquad (3.2)$$

The concentration of D_b can be calculated from the equilibrium between free and bound species. The equilibrium constant K_d is given by the following equation;

$$K_d = \frac{([D_t]-[D_b])([L_t]-[D_b])}{D_b} \qquad (3.3)$$

where L_t is the total concentration of added protein, and D_t the total dye concentration.

Solving equation 3.3 for D_b:

$$D_b = \tfrac{1}{2}\{[D_f]+K_d+[L_t]\} - \sqrt{(D_f+K_d+L_t)^2 - 4(D_f L_t)} \qquad (3.4)$$

By substituting this expression for D_b in equation 3.2, the absorbance change can be related to K_d, D_f and L_t.

$$A_{595} = (E_b - E_f)\tfrac{1}{2}(D_f + K_d + L_t) - \sqrt{(D_f+K_d+L_t)^2 - 4(D_f L_t)} \qquad (3.5)$$

Equation (3.5) can be fitted to experimental data using non-linear regression to provide least squares estimates of L_b, L_f, D_f and K_d. Mayes (1992a) found that the maximum dye availability on blue dextran conjugates was 27%. Figures 3.8 shows the fit of blue HE-starch titration with lysozyme blue HE-starch had a dye availability of 26%. This shows that blue HE-starch has the potential of being a tentacle polymer. The HE-starch has an abundance of primary hydroxyls (6.2 every 10 glucose units, Oxford nutrition). This may allow a greater amount of blue HE-starch to be immobilised onto epoxy PVA-polystyrene, than was achieved with immobilised blue dextran PVA-polystyrene.

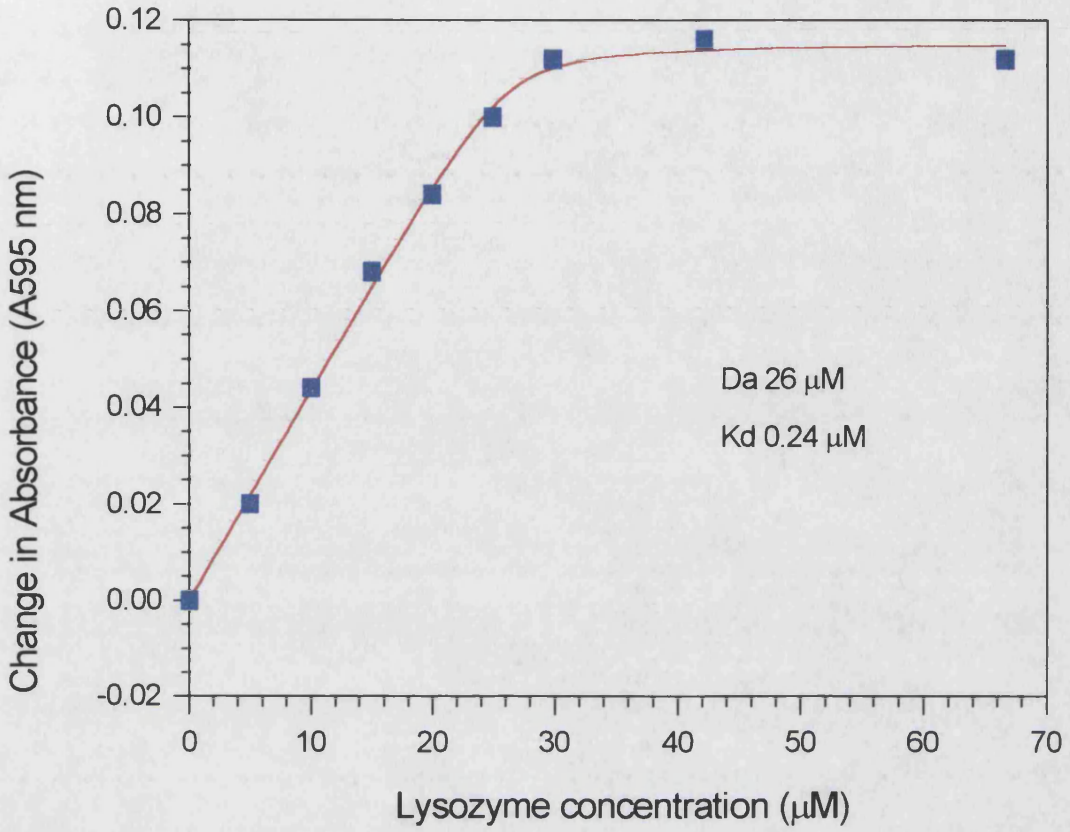


Figure 3.8: *Mutual depletion model fit to the titration of blue HE-starch with lysozyme*

The change in absorbance at 595 nm was determined from the difference spectra, of the titration of blue-HE-starch with lysozyme. The data curve was fitted with a mutual depletion model (equation 3.5). The dye availability (Da) was 26 μM and the dissociation constant (Kd) was 0.24 μM.

The difference spectra obtained for the titration of blue HE-starch and lactate dehydrogenase (LDH) displayed only one isosbestic point (Figure 3.9). The initial absorbance started to increase with each addition of protein, to the extent that at higher concentrations of LDH the spectra did not superimpose. This effect could also be observed in the cuvette where the solution appeared to be increasing in turbidity. The turbidity was due to the LDH-blue HE-starch complex precipitating due to the increasing intermolecular interactions between LDH and the dye ligands attached to HE-starch. This phenomenon is also observed when blue dextran is titrated with lysozyme (Mayes 1990) and when blue HE-starch is titrated with lysozyme (Figure 3.7). The saturation curve was obtained by the difference between the peak at 640 nm and the trough at 705 nm. The saturation curve has a sigmoidal shape and was fitted to equation 3.5. The model gave a dye availability of 45% (Figure 3.10) which is twice the

value obtained for the blue HE-starch titration with lysozyme. This value is higher than blue dextran which has a dye availability of 27%. The higher dye availability could be due to a more specific binding interaction between LDH and the dye because the dye binds at the co-factor binding site. The dye-lysozyme is mainly an ionic interaction between the sulphonate groups of the dye and the positively charged amino acid residues of lysozyme.

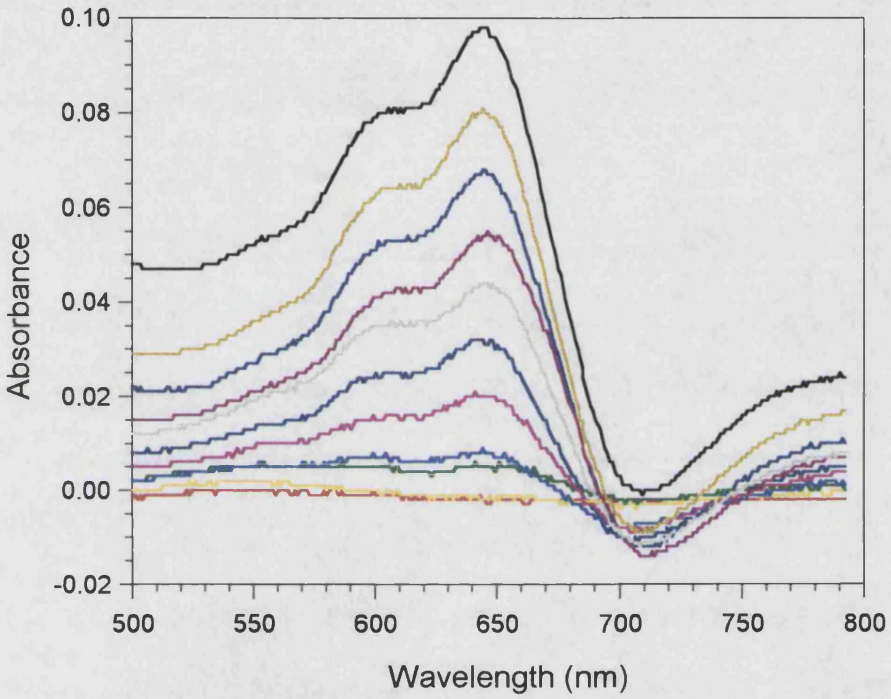


Figure 3.9: *Spectral titration of blue HE-starch with LDH*

Sample and reference cuvettes each containing a solution of 50 μM (with respect to dye) conjugate in 50 mM sodium phosphate pH 7.9, were placed in a Cecil 6000 spectrophotometer. Small volumes of 0.25 mM (with respect to subunits) lactate dehydrogenase was added to the sample cuvette, and equal volumes of buffer were added to the reference cuvette. The cuvette contents were mixed with a small paddle which was placed in situ, without blocking the light path. The change in absorbance was measured after each lactate dehydrogenase addition, by scanning from 500-700 nm. This process was repeated with increasing amounts of lactate dehydrogenase as the magnitude of the increase in the difference spectrum decreased. The titration was stopped when there was no further increase in the magnitude of the difference spectrum. Lactate dehydrogenase was used in the concentration range 0-50 μM.

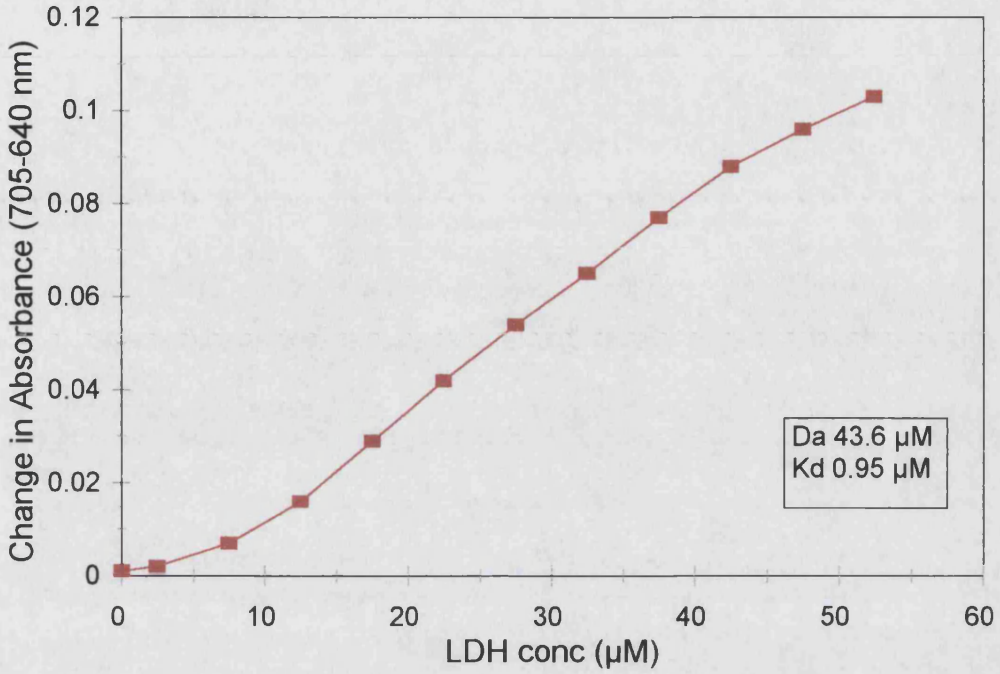
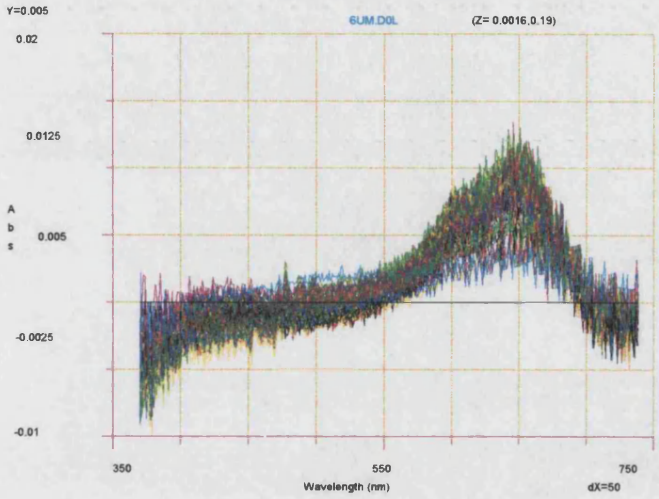


Figure 3.10: *Mutual depletion model fit to the titration of blue HE-starch with LDH*

The saturation curve was obtained from the difference spectra, from the titration with LDH. The curve has a sigmoidal shape and was fitted to a mutual depletion model. The model gave a dye availability (Da) of 45 μM and a dissociation constant (K_d) of 0.95 μM.

The stopped flow experiments with blue HE-starch and LDH were similar to the difference spectra obtained for the spectral titration (Figure 3.8). The experiments were carried out at two different LDH concentrations (120 μM and 60 μM with respect to subunits). The shoulder was more pronounced at the higher LDH concentration. This is due to the concentration of LDH in excess and the difference spectra is more pronounced. A cross section of the peak at 640 nm was taken and was averaged, and the curve was fitted to a single exponential model.

A)



B)

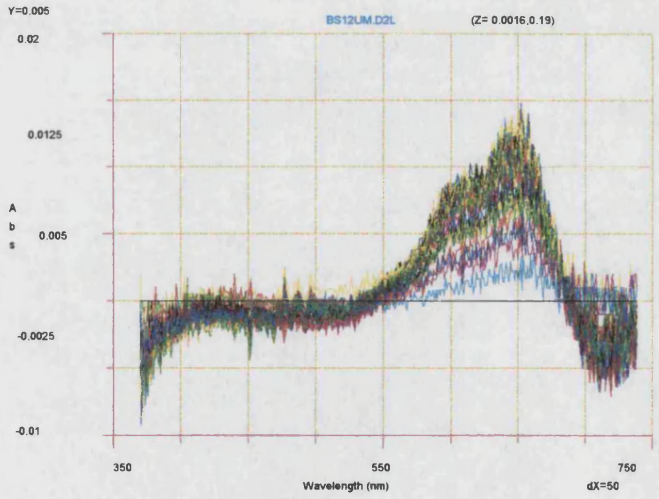


Figure 3.11: *Stopped flow experiments of blue HE-starch conjugate and LDH at A) 60 μM and B) 120 μM* Syringes were filled with 240 μM or 120μM (with respect to subunits) lactate dehydrogenase in 100 mM sodium phosphate buffer pH 7.9 and 100 μM Blue HE-starch conjugate (with respect to dye). Injection was carried with a pneumatic ram which achieved efficient mixing, and very low dead times.

exponential model (figures 3.12a and 3.12b). The fits were reasonable but there wes not enough data in the initial part of the curve for the model to describe the curve fully, especially when the protein was in excess (figure 3.12b). To obtain more data for the initial part of the curve. Single wavelength stopped flow experiments would need to be carried out at 4°C which would slow the reaction rate. However, the poor chromatographic performance of immobilised blue HE-starch obviated the need for single wavelength experiments to be carried out (section 3.8.3.).

A)

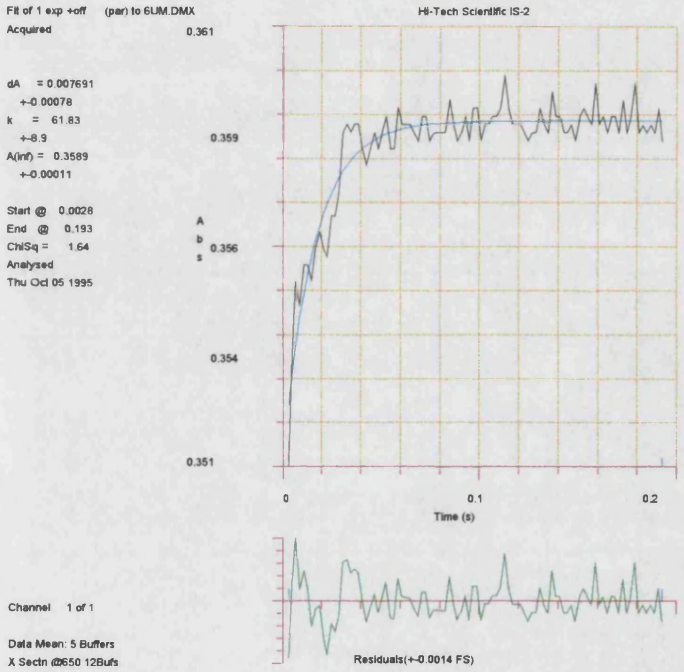


Figure 3.12a: *Single exponential model fit to stopped flow experiment with blue HE-starch (50μM with respect to dye) and LDH* (60 μM, with respect to subunits).

The data were taken from a cross section of a peak at 640 nm from the difference spectrum obtained (figure 3.11a) and was fitted with the computer software supplied with the instrument.

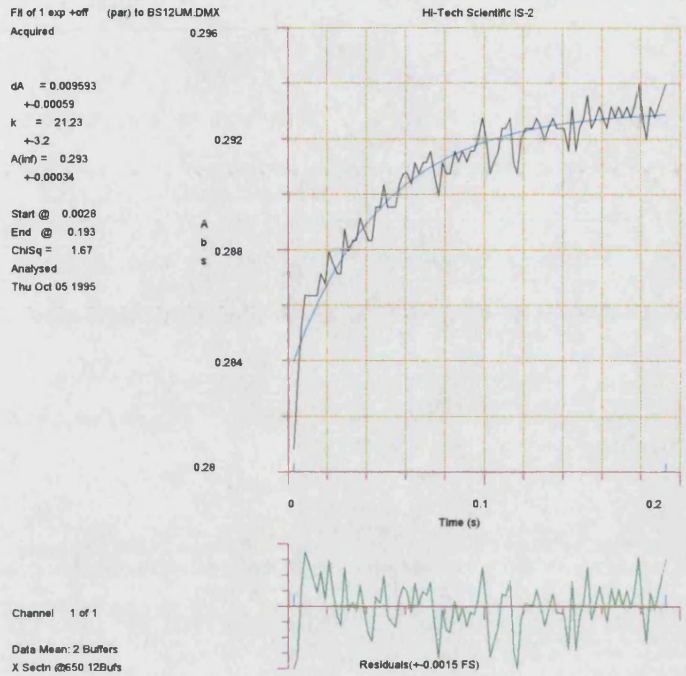


Figure 3.12b: *Single exponential model fit to stopped flow experiment with blue HE-starch (50μM with respect to dye) and LDH* (120 μM, with respect to subunits).

The data were taken from a cross section of a peak at 640 nm from the difference spectrum obtained (Figure 3.11b) and was fitted with the computer software supplied with the instrument. The initial part of the reaction is rapid and hence there is not enough data for the model to fit the data closely.

3.8.3. Equilibrium stirred batch experiments using immobilised blue HE-starch PVA-Polystyrene

A low amount of blue HE-starch was immobilised onto the PVA-polystyrene which resulted in a low amount of immobilised dye (80 nmole ml^{-1}). The low amount of bound polymer is probably due to multipoint attachment of the same molecule to several epoxy groups, thus reducing the total number of polymer chains being immobilised. Lysozyme was used as a model protein to determine the binding capacity and the dissociation constant. The dissociation constant (K_d) was 0.11 μM and the maximum binding capacity (q_m) was 710 $\mu g ml^{-1}$ for lysozyme (figure 3.8). The capacity was quite low as expected due to the low amount of dye immobilised as was found with immobilised blue dextran PVA-polystyrene. A K_d of 10^{-6} M shows that the blue HE-starch has a high affinity for the lysozyme which is similar to the value obtained for immobilised blue

dextran polystyrene. Thus blue HE-starch PVA-polystyrene support did not appear to differ greatly from immobilised blue dextran.

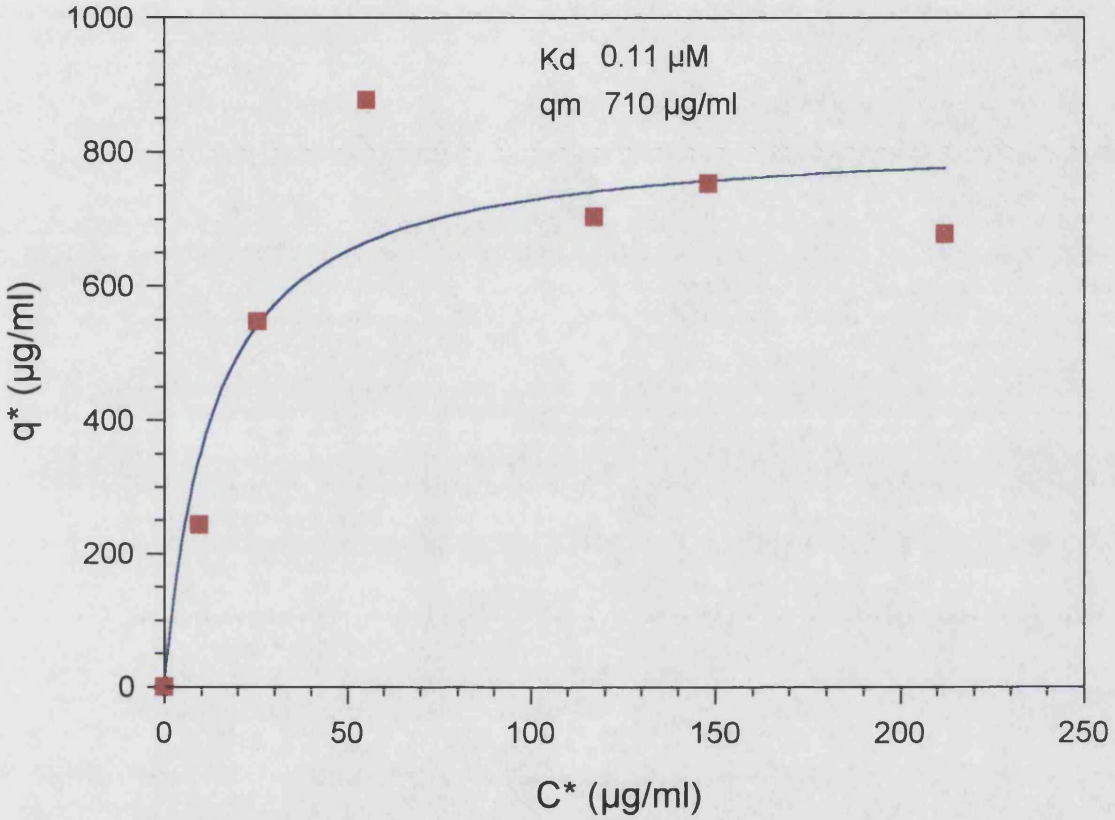


Figure 3.13: *Equilibrium binding isotherm for immobilised blue HE-starch on PVA-polystyrene with lysozyme.*

This apparatus was previously described by Horseman *et al.*, (1986). A water jacketed vessel maintained at 25°C, was set up with an over head stirrer and a 20 µm porosity filter. The filter led to an external loop which contained a Cecil 272 spectrophotometer equipped with a UV flow cell and a Peristaltic pump. Buffer (10 mM Tris 50 mM NaCl, 50 ml) containing lysozyme (conc range 15-500 µg ml^{-1}) was pumped around the system at a fast flowrate to reduce the response time. At time zero 1.4 g of sintered dried adsorbent was added to the vessel and the trace was recorded with a computer data compiler. The amount of protein bound could be determined by the difference between the two plateau's, or by using a computer model of Chase (1984).

This was disappointing as it was thought that the presence of primary hydroxyls at high density would increase the amount of polymer immobilised since primary hydroxyls are more reactive than secondary hydroxyls (Uy and Wold, 1977). The dye would be coupled to a large number of these substituted side chains but there still would be a sufficient number of free hydroxyl groups free to react with epoxy groups. These results highlight the fact that the hydroxyl groups are not the most efficient nucleophiles. Hence many of the synthetic steps required quite harsh conditions.

3.9. HYDROXYETHYL-PVA

3.9.1. Hydroxyethylation of polyvinyl alcohol

Primary hydroxylation of the polymer aids solubilisation of the derivitised polymer, Cohen *et al* (1953) found that the primary hydroxylated PVA was more readily water soluble than PVA. However these workers used ethylene oxide under pressure to effect primary hydroxylation. As this is a hazardous procedure the method of Chun *et al* (1990) was adopted. This involves reaction of PVA with halocarboxylic acids in the presence of NaOH. This procedure was adapted for use with bromoethanol. In this way primary hydroxylation was achieved. The resulting polymer was amber coloured as described by Cohen and was readily water soluble (figure 3.14).

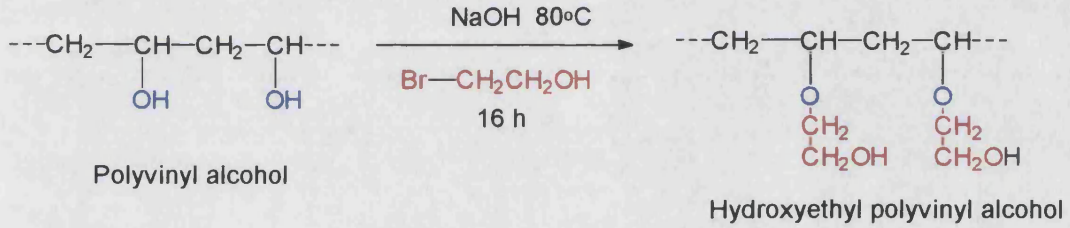


Figure 3.14: *Reaction scheme for the synthesis of hydroxyethyl polyvinyl alcohol*

Primary hydroxylation of PVA was necessary because Cibacron blue could not be coupled to the PVA polymer using the standard coupling conditions. This is due primarily to the unreactivity of the secondary hydroxyl groups of polyvinyl alcohol. An abundance of primary hydroxyls could improve dye coupling and could ultimately increase the amount polymer bound to the support.

However, the derivitised Cibacron blue-PVA was difficult to dissolve even in hot water. The reason for this is unclear but never the less polyvinyl alcohol was immobilised to PVA-polystyrene. This produced a dye loading of 15 nmole dye ml^{-1}. The very low ligand concentration led to a low binding capacity of 50 $\mu g\ ml^{-1}$ using lysozyme. Thus PVA was not suitable to be used as a tentacle polymer.

3.10. BLUE POLYETHYLENE IMINE

3.10.1. Synthesis of blue-PEI

Polyethylene imine (PEI) was chosen as an alternative synthetic polymer because the polymer contains primary and secondary amine groups which are good nucleophiles (figure 3.15). The polymer seemed to behave in exactly the same manner as dextran and HE-starch, but at the dialysis stage it was noticed that the blue PEI appeared to be a very fine particulate suspension, which was confirmed when the polymer was freeze dried. The problems became more evident when the dye loading on the PEI became >2 mol dye/mol polymer. The derivitised polymer was insoluble in the coupling buffer (188 mM Na_2CO_3). Water insoluble ligands which are used for affinity chromatography are dissolved using a water miscible solvent such as DMF, dioxan or DMSO upto 50% (v/v) (Pharmacia). Blue-PEI was partially soluble in 50% (v/v) DMF/water.

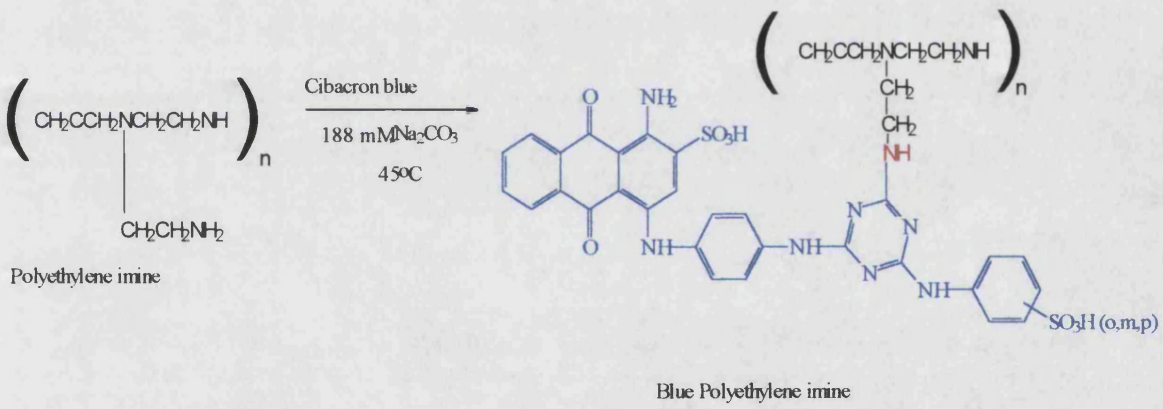


Figure 3.15: *Synthesis of blue polyethylene imine (blue-PEI)*

3.10.2. Deviation from Beer-Lambert Law

The blue-PEI could only be dissolved in 50% (v/v) DMF/water (v/v). This meant that the usual method of determination of dye loading (acid hydrolysis in 6 M HCl) was not suitable. This was unfortunate because in the presence of acid dye stacking does not occur (Mayes, 1992). Dye stacking causes a deviation from the Beer Lambert law when the concentrations of dye in water is >5 μM. The presence of DMF however, might prevent the hydrophobic dye-dye interactions and thus avoiding stacking. The result shown in figure 3.16 indicates the Beer Lambert relationship is applicable. The extinction coefficient of Cibacron blue in 50% (v/v) DMF/water was estimated to be

13, 052 M^{-1} cm^{-1} at 610 nm. This value is comparable to the value in water $\varepsilon_{610\ nm}$ 13, 600 M^{-1} cm^{-1} (Thompson and Stegwallen 1976).

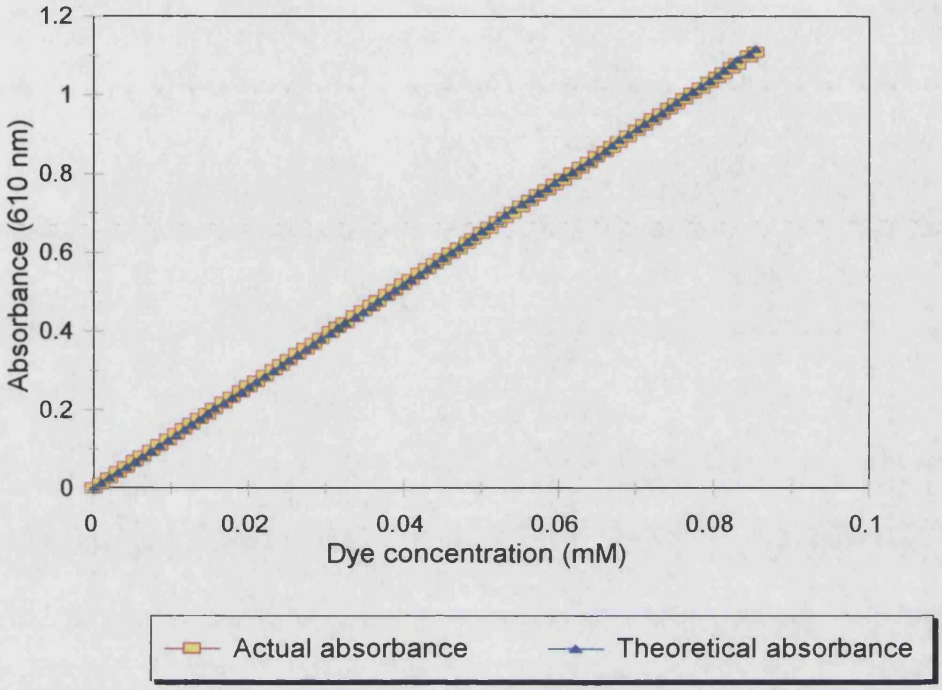


Figure 3.16: *The adherence of Cibacron blue in DMF/water (50% (v/v) to the Beer-Lambert law.*

DMF/water (50% (v/v), 2 ml was placed in a quartz cuvette and placed in a dual beam Cecil 660 spectrophotometer. Additions of a concentrated stock solution of dye in 50% (v/v) DMF/water were made to the cuvette. The contents were mixed with a paddle in the cuvette. The absorbance at 610 nm was recorded as further additions of dye were made until the absorbance reached a value of 1.

3.10.3. Synthesis of immobilised blue-PEI onto epoxy PVA-polystyrene

In the absence of added base the solubilised blue-PEI-polymer did not react with the epoxy activated support. The organic base employed was tetramethylammonium hydroxide. This allowed the blue-PEI to react with the epoxy activated PVA-polystyrene. Blue-PEI which had a dye loading of 19 mol of dye/mol of polymer was chosen for immobilisation because of the very high dye loading (equivalent to 360 mol dye/mol polymer if based on polymer of 0.5x 10^6 Da). However, the amount of immobilised dye was still only 100 nmole dye ml^{-1} which was only slightly higher than immobilised blue HE-starch (80 nmole dye ml^{-1}). This might be due to many of the reactive amine groups being coupled with dye, and therefore not available for coupling. The high dye loading might have also have the same effect as dextran, causing the

hydrodynamic radius to shrink and reduce the amount of free amine groups available for binding (section 3.7.2).

3.10.4. Equilibrium stirred batch experiments on immobilised blue-PEI PVA-polystyrene

The chromatographic performance of immobilised blue-PEI was disappointing. The protein binding being negligible at pH 7.9 which was the standard conditions for binding of LDH. PEI consists of primary, secondary and tertiary amino groups (ratio 1:1:1, Geckler *et al.*, 1980). The primary amine groups are most likely to be coupled to dye molecules because primary amines are stronger nucleophiles than secondary and tertiary amines. Thus any remaining amine groups will act as weak anion exchange groups (pK_a 7-9, Helfferich, 1962). The effect of pH was investigated to determine whether there was an influence on protein binding.

Binding capacity improved as the pH decreased, but protein desorption occurred at pH 7 and pH 6. At pH 5 there was no desorption (figure 3.17). The pI of LDH is 8.1 and will have a net positive charge as the pH decreases. This may promote ionic interactions between the negatively charged sulphonate groups of the dye molecule and positively charged LDH causing an increase in binding. However, the underivitised amino groups are becoming progressively charged on the PEI molecule which may repel LDH molecules. This will cause a disruption of the ionic interactions between LDH and dye and desorption of the protein which may explain the traces at pH 6 and 7 (figure 3.17). At pH 5 the tertiary structure of LDH may become partially disrupted and expose more hydrophobic sites on the molecule. The greater amount of hydrophobic surface will help strengthen the binding between LDH and dye molecules, since dye molecules interact through a combination of hydrophobic and ionic interactions (Turner, 1981). The increased hydrophobic binding may be strong enough to overcome the repulsive forces from the protonated amine groups of PEI. Thus reducing the desorption of LDH which is observed at pH 6 and 7.

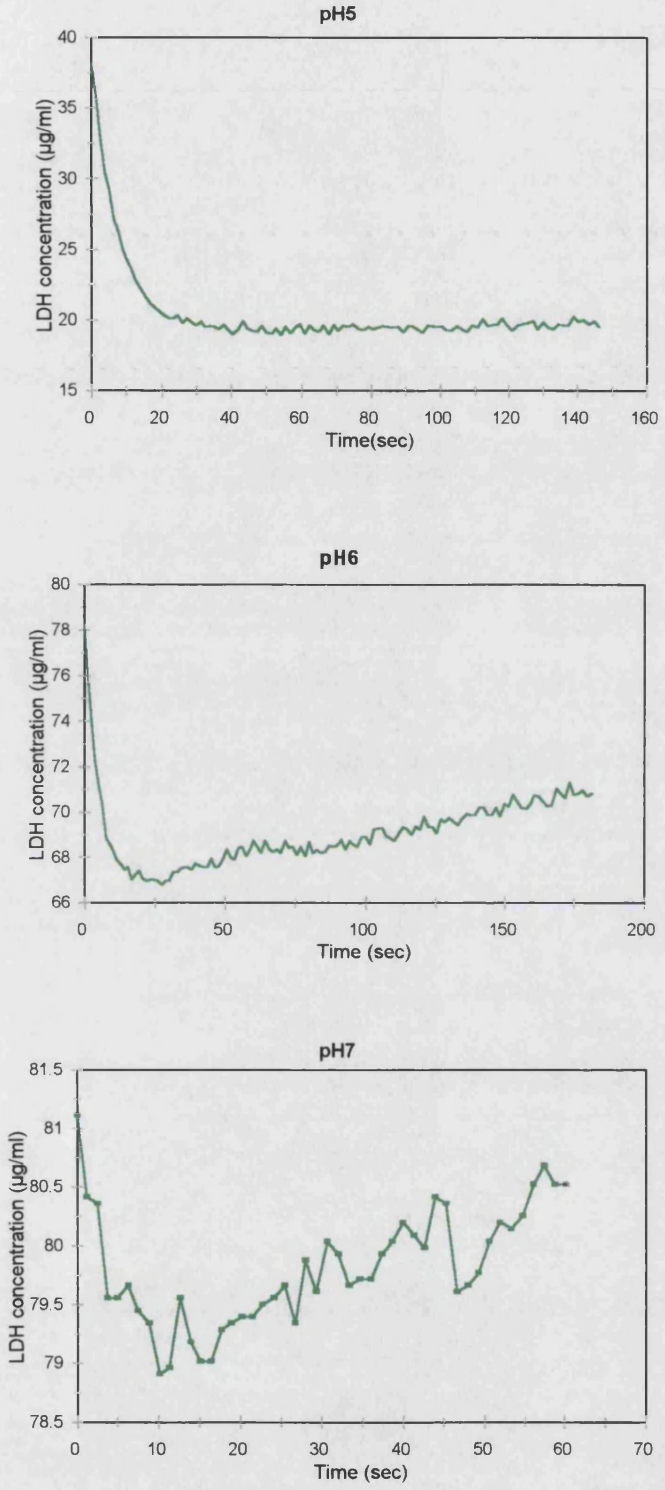


Figure 3.17: Adsorption of LDH to immobilised blue-PEI as a function of pH.
A water jacketed vessel maintained at 25°C, was set up with an overhead stirrer and a 20 µm porosity filter. The filter led to an external loop which contained a Cecil 272 spectrophotometer equipped with a UV flow cell and a Peristaltic pump. Buffer which was 50 mM sodium acetate pH 5, sodium phosphate pH 6 or sodium phosphate pH 7, (25 ml) which contained LDH, was pumped around the system at a fast flowrate to reduce the response time. At time zero 1.4 g of sintered dried adsorbent was added to the vessel and the trace was computer logged.

3.11. CONCLUSIONS

PVA-polystyrene beads have been shown to be a stable support for the synthesis of tentacle affinity supports. Dye conjugates such as blue dextran and blue HE-starch were immobilised onto PVA-polystyrene beads. However, it was not possible to immobilise more than 400 μg ml^{-1} of polymer on the bead surface, despite using a variety of activation procedures. The major problem was probably due to hydroxyl groups being weak nucleophiles. As a consequence the more reactive hydroxyl groups are coupled to dye molecules. Another possible explanation might be due to the increased dye loading. The proximity of neighbouring dye molecules is decreased, resulting in an increase in hydrophobic dye-dye interactions. The interactions cause the polymer molecules to compact, reducing the hydrodynamic radius. Thus decreasing the number of free hydroxyls available to interact with the epoxide groups on the bead surface.

In spite of these problems tentacle supports were synthesised. Immobilised blue dextran had a binding capacity of 0.34 mg ml^{-1} and a K_d of 0.6 μM which are similar to the K_d values obtained for blue dextran in solution (Mayes *et al.*, 1992). From the results obtained, blue dextrans affinity for protein is not altered significantly by immobilisation. Immobilised blue HE-starch had a protein binding capacity of 0.7 mg ml^{-1} and a K_d of 0.11 μM. Spectral titration's of blue HE-starch were promising, with dye availability's of 26% for lysozyme and 44% for LDH. However, the poor chromatographic performance of immobilised blue HE-starch meant that further characterisation of blue HE-starch in free solution was not required.

The synthetic polymers PVA and PEI were also immobilised in similar amounts (200 and 500 μgml^{-1} respectively) to blue dextran and HE-starch. Hence the immobilised dye concentration was low (15 and 100 nmole dye ml^{-1} respectively). The protein binding capacity was disappointingly only 50 μg ml^{-1} for immobilised blue HE-PVA. Protein binding for immobilised blue-PEI was pH dependent with maximum binding occurring at pH 5. The charged backbone of PEI appeared to be interfering with the dye ligands ability to interact with protein. PEI was only soluble in 50% (v/v) DMF/water, thus spectral titrations and stopped flow experiments were not an available option to characterise the blue-PEI in solution.

The very low ligand densities displayed by all the immobilised polymers indicate that the possibility of using pre-characterised soluble dye conjugates for tentacles is not feasible. Therefore other alternatives must be investigated for suitable polymer tentacles.

CHAPTER FOUR

BLUE DEXTRAN PVA-POLYSTYRENE

4.1. INTRODUCTION

The low amount of immobilised dye-polymer conjugates led to a different approach (sections 3.7.1-3.10.4). It was noted that the less dye-substituted dextran was immobilised in larger amounts (400 $\mu g ml^{-1}$, dextran immobilised) than the more highly dye-substituted dextran (150 $\mu g ml^{-1}$, dextran immobilised). It will be interesting to determine whether a larger amount of underivitised dextran can be immobilised. It may be possible to immobilise dye ligands to concentrations approaching that of the commercial supports. Blue Sepharose CL-6B has a dye concentration of 4 $\mu mole\ ml^{-1}$.

Similar work has been carried out by Santarelli *et al.* (1990) and Girot *et al.* (1990) where silica beads were coated with dextran, cross linked with 1,4-butane diglycidal ether and used as affinity supports. The dextran coating prevented proteins being non-specifically adsorbed and protected silica from being dissolved by NaOH.

4.2. EXPERIMENTAL

4.2.1. Time course for the immobilisation of dextran to PVA-polystyrene

PVA-polystyrene beads (10 g) were placed in 100 ml of 1 M NaOH and heated to 60°C. 2-Hydroxyethyl methacrylate (6.1 ml, 0.5 M) was added to the suspension and stirred at 60°C for 2 hours. The beads were washed with 200 ml of water and suspended in 17 ml of 2 M NaOH containing sodium borohydride (2 mg ml^{-1}), 8.5 ml of epichlorohydrin was added dropwise and the suspension was stirred overnight. The epoxy activated beads were washed sequentially with water (500 ml), acetone (100 ml) and water (500 ml).

The epoxy activated PVA-polystyrene beads were placed in 30 ml of 0.25 M NaOH which contained dextran (250 mg ml^{-1}) and sodium borohydride (2 mg ml^{-1}). The suspension was stirred at 50°C in a glass beaker, samples (2.5 ml) were removed at timed intervals and washed with water on a sintered glass funnel. The samples were dried in an

oven at 80°C overnight and were assayed for the amount of immobilised dextran using the phenol sulphuric acid assay (section 2.3.4).

4.2.2. Improved coupling of dextran onto PVA-polystyrene

Epoxy activated PVA-polystyrene beads (10 g) were prepared as described in section 4.2.1. The beads were added to 100 ml of 0.25 M NaOH which contained dextran (250 mg ml^{-1}) and sodium borohydride (2 mg ml^{-1}) and stirred for 5 hours at 50°C. The beads were washed with 1 L of water, a sample (50 mg) was removed for dextran determination (section 2.3.4). Cibacron blue was immobilised using the revised method of Lowe and Pearson (1984). Cibacron blue (200 mg) was dissolved in 100 ml of water, dextran PVA-polystyrene (10 g) was added and stirred for 10 minutes. NaCl (5.9 g, 1M) and Na_2CO_3 (21.2 g, 2 M) was added and the suspension was stirred at 60°C for 4 hours. The beads were washed with water until the washings were virtually colourless. The beads were packed into a 25 ml column and washed with 200 ml of 1 M NaCl, 400 ml of 50 % (v/v) ethanol/water, 100 ml of 1 M NaCl and finally with 100 ml of 50 mM sodium phosphate buffer pH 7.9. The beads were then stored in sodium phosphate buffer with a few crystals of sodium azide at 4°C.

4.2.3. Synthesis of a range of dye loadings

A range of dye loadings were obtained by following the procedure described in section 4.2.2., except that the amount of dye in the coupling solution was varied as detailed in table 4.1:

Table 4.1: Summary of the synthesis of PVA-polystyrene supports with different dye loadings

PVA-polystyrene code	Dye concentration (mg ml^{-1})	Comments
PV2	25	single dye addition with extensive washing, with water and 50% (v/v) ethanol
PV3	i) 4 ii) 10	extensive washing with water, followed by second coupling reaction
PV4	i) 10 ii) 10 iii) 5	extensive washing with water between each coupling reaction

4.2.4: Dye loading determination of the blue dextran PVA-polystyrene

This method was adapted from a dextranase activity assay described by Janson and Porath (1966). Dry blue dextran PVA-polystyrene (700 mg, section 4.2.2) was placed in a centrifuge tube (15 ml) which contained 2 ml of 0.1 M sodium phosphate buffer pH 6.0 and a magnetic follower. Dextranase (11 units, IU) in 10 mM sodium acetate buffer pH 5.5, was added and the tube was incubated at 37°C. The reaction was stopped at timed intervals, by centrifugation for 1 minute. The absorbance of the supernatant was measured at 595 nm against a buffer blank. An extinction coefficient of 10, 500 M^{-1} cm^{-1} (Chambers 1977) was used to determine the dye concentration. The supernatant was placed back into the reaction mixture and the tube was reincubated at 37°C.

4.2.5. Acid hydrolysis of blue dextran PVA-polystyrene

The dye loading of the support was also determined using acid hydrolysis, as described in section 3.2.3. In the case of blue dextran supports which had a higher concentration of immobilised dye (PV3 and PV4, table 4.1) after centrifugation, the samples were reincubated with 2 ml of 6M HCl for 2 hours and then treated as described previously. This was repeated several times until the absorbance of the supernatant reached a value of 0.001 at 541 nm.

4.2.6. Equilibrium stirred batch experiments

Experiments were carried out as described in section 3.3.7. with LDH in the concentration range 36-550 μM.

4.2.7. Pressure flowrate characteristics of blue dextran PVA-polystyrene and blue Sepharose CL-6B

Blue dextran PVA polystyrene (1 ml) was packed into a small column (0.66 x 3 cm), and the bed was subjected to steadily increasing linear flow rates (88-1200 cm hr^{-1}) using a peristaltic pump. A pressure gauge was located at the column inlet and the resulting increase in back pressure was measured. The experiment was repeated with Blue Sepharose CL-6B using the same conditions.

4.2.8. Purification of fumarase using blue dextran PVA polystyrene

Chicken liver fumarase was a gift from Sigma Chemical Company. The crude sample was a 60-80% ammonium sulphate cut. All operations were carried out at 4°C unless otherwise stated.

A sample (20 ml) of crude fumarase was centrifuged at 17,500 g, the supernatant discarded and the pellet resuspended in a minimum volume of buffer A (10 mM Tris 14 mM β-mercaptoethanol, pH 7.3). The resulting suspension was dialysed against 1L of buffer A overnight. The dialysed was centrifuged at 17,500 g. The supernatant was passed through a 0.2 μm membrane filter and loaded onto a 4 ml column of Blue dextran PVA-polystyrene equilibrated with buffer A at a flowrate of 0.5 ml min^{-1}. The column was washed with buffer A until the absorbance of the eluate at 280 nm was below 0.01. Bound fumarase was eluted with 10 mM L-malic acid in buffer A. The fractions (0.5 ml) containing significant activity were pooled. The purified protein was analysed by SDS PAGE on 10% acrylamide gels (section 2.3.8).

4.2.9 Assay of fumarase activity

L-Malic acid (50 mM, 2.26 g) was dissolved in potassium phosphate buffer (0.1 M, 290 ml) the pH was readjusted to 7.6 with 1 M KOH. All fractions to be assayed were kept on ice. The assay solution (2.9 ml) was pipetted into a quartz cuvette at 25°C and 100 µl of sample was added. The contents were mixed briefly using a piece of Nesco® film to cover the cuvette and the change in absorbance at 240 nm was monitored using a Cecil 660 Spectrophotometer.

4.3. RESULTS AND DISCUSSION

4.3.1. Timecourse for the immobilisation of dextran

The time course was followed in order to allow optimisation of the amount of dextran immobilised on PVA-polystyrene beads. 2-Hydroxyethyl methacrylate (HEMA) was introduced into the PVA-polystyrene by Michael's addition (Pittfield 1994). HEMA was used instead of N-(Hydroxy methyl) acrylamide (section 3.2.5) because HEMA is one of the most alkali stable monomers (Hermanson *et al* 1992b). This is especially important because of the high pH required for the activation of the PVA-polystyrene beads with epichlorohydrin and coupling of dextran and dye. The resulting beads now have primary hydroxyls which are easier to derivatise with epichlorohydrin (Porath and Olin, 1983), figure 4.1 shows the reaction scheme.

Figure 4.2 shows that the coupling of dextran to epoxy polystyrene is slow with the reaction taking 5 hours to reach completion. A high concentration of dextran was used to promote single point attachment of dextran molecules rather than multipoint attachment of a dextran molecule via several epoxy groups. This was to encourage dextran to immobilised in a tentacle form as opposed to dextran molecules being stretched around the surface of the bead (Kitigawa, 1988). The time course showed that about 800 $\mu g\ ml^{-1}$ of dextran was immobilised, which was twice as much bound as the lower loaded blue dextran conjugate (400 $\mu g\ ml^{-1}$). The greater amount of dextran bound could be attributed to there being a much greater availability of free primary hydroxyls which have the potential of reacting with the epoxy groups.

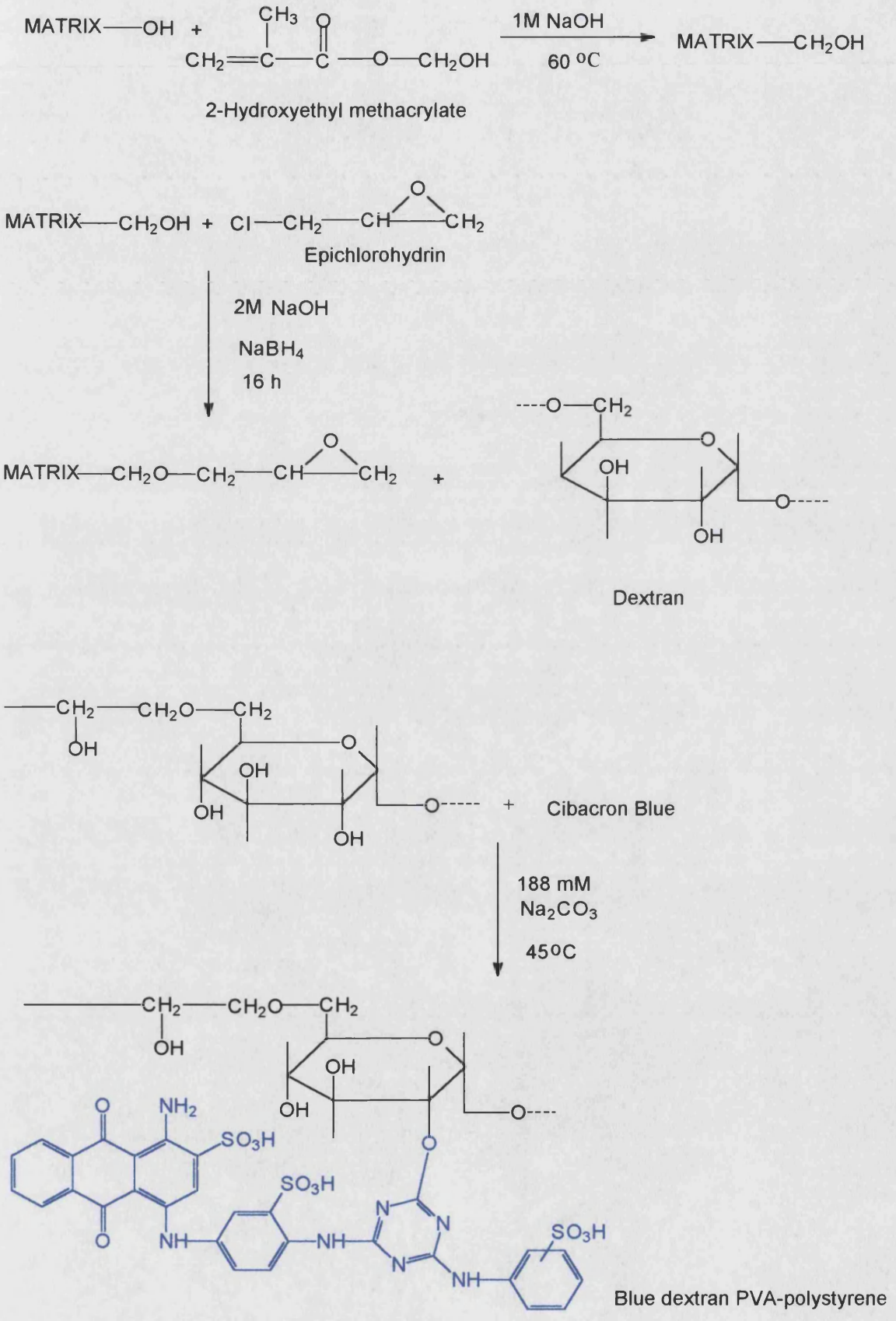


***Figure 4.1**: Reaction scheme for the synthesis of blue dextran PVA-polystyrene*

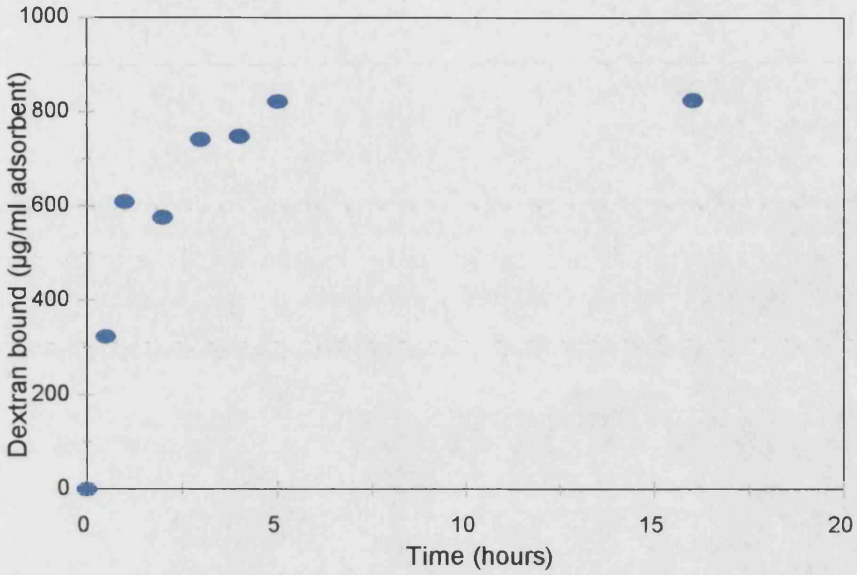


Figure 4.2: *Time course for the immobilisation of dextran onto epoxy activated PVA-polystyrene.*

Epoxy activated polystyrene beads were placed in 30 ml of 0.25 M NaOH which contained dextran (250 mg ml^{-1}) and sodium borohydride (2 mg ml^{-1}). The suspension was stirred at 50°C in a glass beaker, samples (2.5 ml) were removed at timed intervals and were washed with water on a sintered glass funnel. The samples were dried in an oven at 80°C overnight, and were assayed for the amount of immobilised dextran using the phenol sulphuric acid assay (section 2.3.4).

4.3.2. Synthesis of blue dextran PVA-polystyrene

Cibacron blue was introduced into the dextran PVA-polystyrene. The colour of the blue dextran PVA-polystyrene beads was darker than that obtained with immobilised blue dextran polymer (section 3.2.4). The immobilised dye could be bound to the dextran and also could be bound to 1,2 diols which are produced by the hydrolysis of epoxy groups (figure 4.1). Thus to determine the dye loading on the dextran only, an endo dextranase was used to hydrolyse the dextran molecule. The results are shown in figure 4.3. The loading was calculated to be 0.2 µmole ml^{-1} which is about twenty fold less than the dye loading of commercial blue Sepharose CL-6B (Pharmacia). The beads were still blue after incubation with dextranase. This could be due to the enzyme becoming sterically hindered and unable to hydrolyse glucose residues of the dextran molecules which are close to the bead surface. The residual colour could also be due to dye molecules coupled to the hydrolysed unreacted epoxy groups on the support surface.

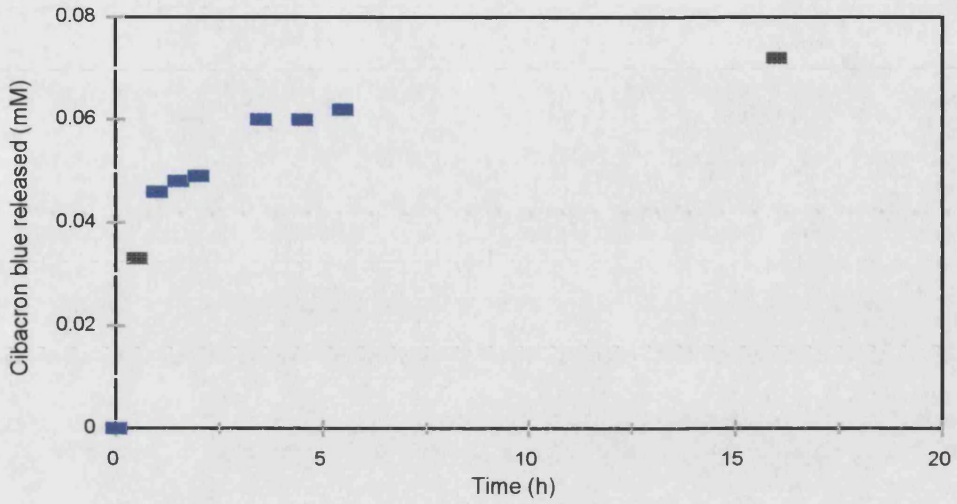


Figure 4.3: *Cibacron blue released by action of dextarnase on blue dextran PVA-polystyrene.*

Dry blue dextran PVA-polystyrene (700 mg, section 4.2.2) was placed in a centrifuge tube, which contained 2 ml of 0.1 M Sodium phosphate buffer 6.0 and a magnetic follower. Dextranase (11 units, IU) in 10 mM sodium acetate buffer pH 5.5, was added and the tube was incubated at 37°C. The reaction was stopped at timed intervals, by centrifugation for 1 min. The absorbance of the supernatant was measured at 595 nm against a buffer blank, an extinction coefficient of 10, 500 M^{-1} cm^{-1} (Chambers 1977) was used to determine the dye concentration. The supernatant was placed back into the reaction mixture, and the tube was reincubated at 37°C.

However, acid hydrolysis of the same beads gave a dye loading of 1.2 μ mole ml^{-1}, the beads were devoid of any colour after the acid hydrolysis. Thus for the most accurate determination of dye loading acid hydrolysis was used. The higher dye loaded beads (PV3 and PV4) were incubated several times with acid to achieve total dye removal. The necessity for repeated incubations with acid gives testament to stability of the immobilised dye linkage. The range of dye loadings of the blue PVA-polystyrene supports is summarised in table 4.2. It was also interesting to note that to achieve higher dye loadings the beads had to be incubated with fresh dye. This phenomenon is also exhibited by dextran in free solution (Mayes *et al.,* 1992). The dye loading tended to reach a constant value only a fresh addition of dye would result in an increase in dye loading. The competing hydrolysis reaction of dye with hydroxide ions reduces the amount of available dye for coupling. Thus the concentration of active dye can only be increased by adding fresh dye to the coupling medium.

Table 4.2: The range of blue dextran PVA-polystyrene supports synthesised

Blue dextran PVA-polystyrene (code)	Immobilised dye concentration (μ mole ml^{-1} adsorbent)
PV1	0.73
PV2	1.2
PV3	6.2
PV4	7

4.3.3 Comparison of porous and tentacle supports.

A binding experiment was carried out to determine if the blue dextran PVA-polystyrene displayed the characteristics of a tentacle support. Figure 4.4 shows the comparison between the porous blue Sepharose CL-6B and blue dextran PVA-polystyrene (PV2). The equilibration time was rapid, which was expected because the beads are non-porous and do not have pore resistances to be overcome. Thus equilibrium is achieved five times faster than the porous blue Sepharose CL-6B. The protein diffuses into the porous bead which slows down the rate of adsorption. Sepharose is made up of a network of pores and the absence of 'through' channels increases the path length for molecular diffusion.

Conversely for the tentacle support, all protein binding takes place externally on the attached dextran polymer chains. There are no pore diffusion resistances or pore wall diffusion resistances to overcome because the PVA-polystyrene beads are non-porous. Only the film resistances around the particles have to be overcome for protein adsorption to occur. As a result protein adsorption is rapid and approaches the equilibrium times of non-porous silica beads 0.5 minutes (Anspach *et al.*, 1989).

A control matrix, dextran PVA-polystyrene was prepared. This support exhibited very low non-specific binding (40 μg ml^{-1}), PV1 had a binding capacity of 2.5 mg ml^{-1} (figure 4.5) which shows that all protein binding on blue PVA-polystyrene is due to the immobilised dye (figure 4.4). The coating of the polystyrene surface with PVA and dextran has totally masked the highly hydrophobic nature of the polystyrene surface.

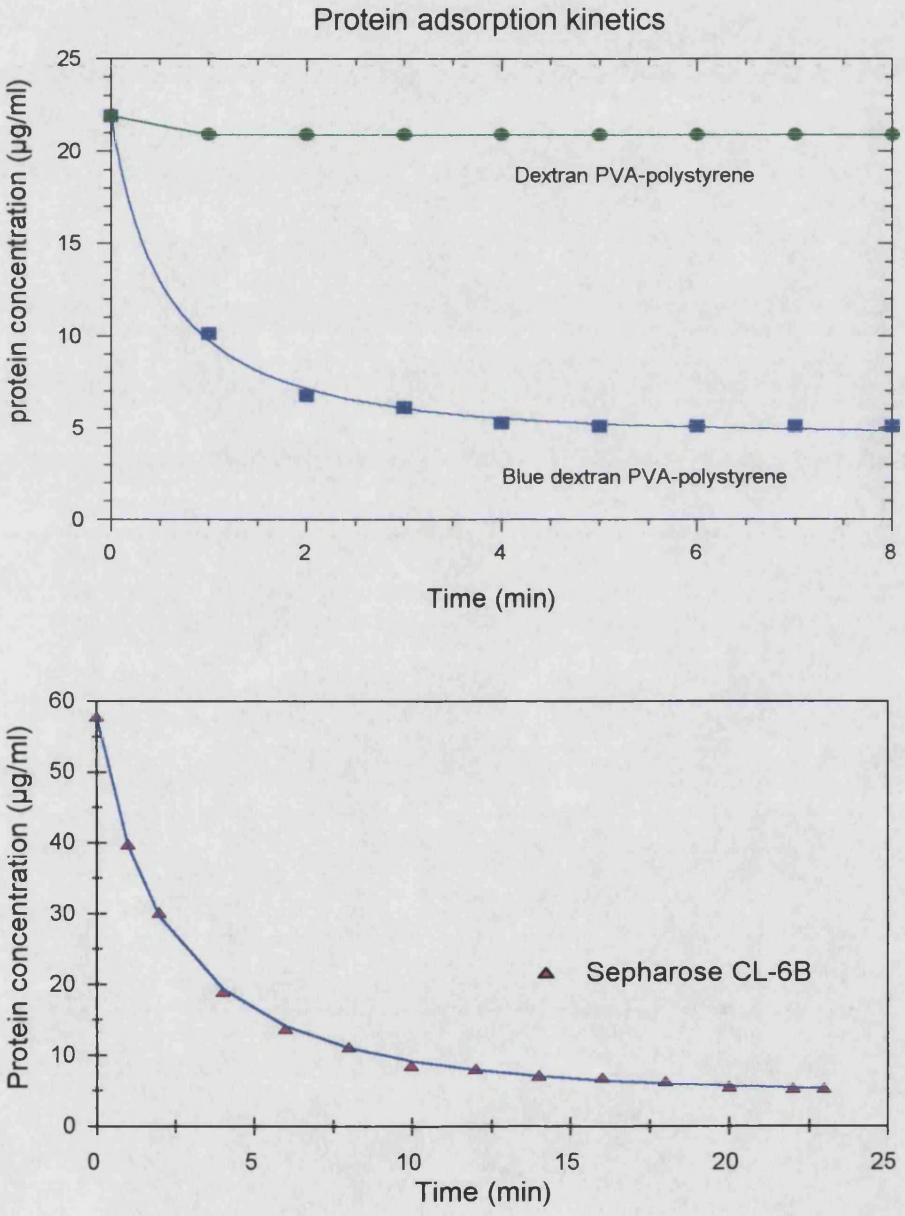


Figure 4.4: *Comparison of the tentacle PVA-polystyrene with blue Sepharose CL- 6B* Kinetic protein adsorption experiments were carried out using the equipment described by Horstmann *et al.*, (1986). A) the blue dextran PVA-polystyrene and the control support dextran PVA-polystyrene, equilibrium is achieved in 4 minutes for the dye support. The control support displays low non-specific binding. B) Porous Blue Sepharose CL-6B equilibrium is achieved in 20 minutes.

4.3.4 Equilibrium batch adsorption

The interaction between a protein (P) and its biospecific ligand (L) is described by the equation:

$$P + L \underset{K_2}{\overset{K_1}{\rightleftharpoons}} P.L \qquad (4.1)$$

where the equilibrium dissociation constant (K_d) is defined by the ratio of (k_2/k_1):

$$K_d = \frac{\kappa_2}{\kappa_1} = \frac{[P].[L]}{[P.L]} \qquad (4.2)$$

Equation 4.2 represents an over simplification of the interaction between a protein and its ligand. The equation holds for homogeneous systems. There are significant contributions from film diffusion resistance outside the bead surface, pore diffusion resistances and resistance from the pore walls. Chase (1984) proposed a simplified assessment for heterogeneous systems based on the use of k_1 and k_2 as lumped parameters which incorporate these resistances. Thus if the rate of adsorption of protein to adsorbent can be defined empirically as a mutual depletion model.

$$\frac{dq}{dt} = \kappa_1 \cdot c \cdot (q_m - q) - \kappa_2 \cdot q \qquad (4.3)$$

Where c represents the concentration of protein in solution, q is the solid phase concentration of the adsorbed protein per unit volume of settled adsorbent, and q_m is the maximum adsorption capacity of the adsorbent.

At equilibrium:

$$\frac{dq^*}{dt} = \kappa_1 . c^* . (q_m - q^*) - \kappa_2 . q^* = 0 \qquad (4.4)$$

where * represents the value at equilibrium. Rearrangement of equation 4.4 describes the relationship between the adsorbed protein (q*) and the concentration of protein (c*) in the soluble phase at equilibrium.

$$q^* = \frac{q_m . c^*}{c^* + K_d} \qquad (4.5)$$

Thus equation 4.5 predicts that the adsorption isotherm is a non linear square hyperbola first described by Langmuir (1916) for the adsorption of gases on solid phases.
For a batch adsorption system the total volume (V_{tot}), with an initial protein concentration (c_o), containing a settled volume of adsorbent (v_{ads}), the concentration of protein (c*) in the soluble phase at equilibrium will be given by:

$$c^* = c_o - \frac{v_{ads} \cdot q^*}{V_{tot}} \qquad (4.6)$$

where q* is the amount of protein immobilised per unit volume of settled adsorbent at equilibrium.

A range of dye loadings was synthesised, in order to determine the effect of ligand density on protein binding capacity (q_m), dissociation constant (k_d) and the forward rate constant (k_1). The experiments were carried out using equilibrium stirred batch apparatus described by Chase (1986). Non-specific protein binding of the support was also determined by using underivitised dextran coated PVA-polystyrene.

Figures 4.5 and 4.6 show the equilibrium adsorption isotherms for the different dye loadings of blue dextran PVA-polystyrene. The data were fitted to equation 4.5. The dye loadings all gave favourable isotherms as described by Langmuir (1916). It is interesting to note that the dissociation constant (K_d) decreases with increasing dye loading, blue-Sepharose also displays this phenomenon (Boyer and Hsu 1992). The decrease in K_d is also exhibited by Blue dextran conjugates in solution, with increasing dye loading (Mayes 1990).

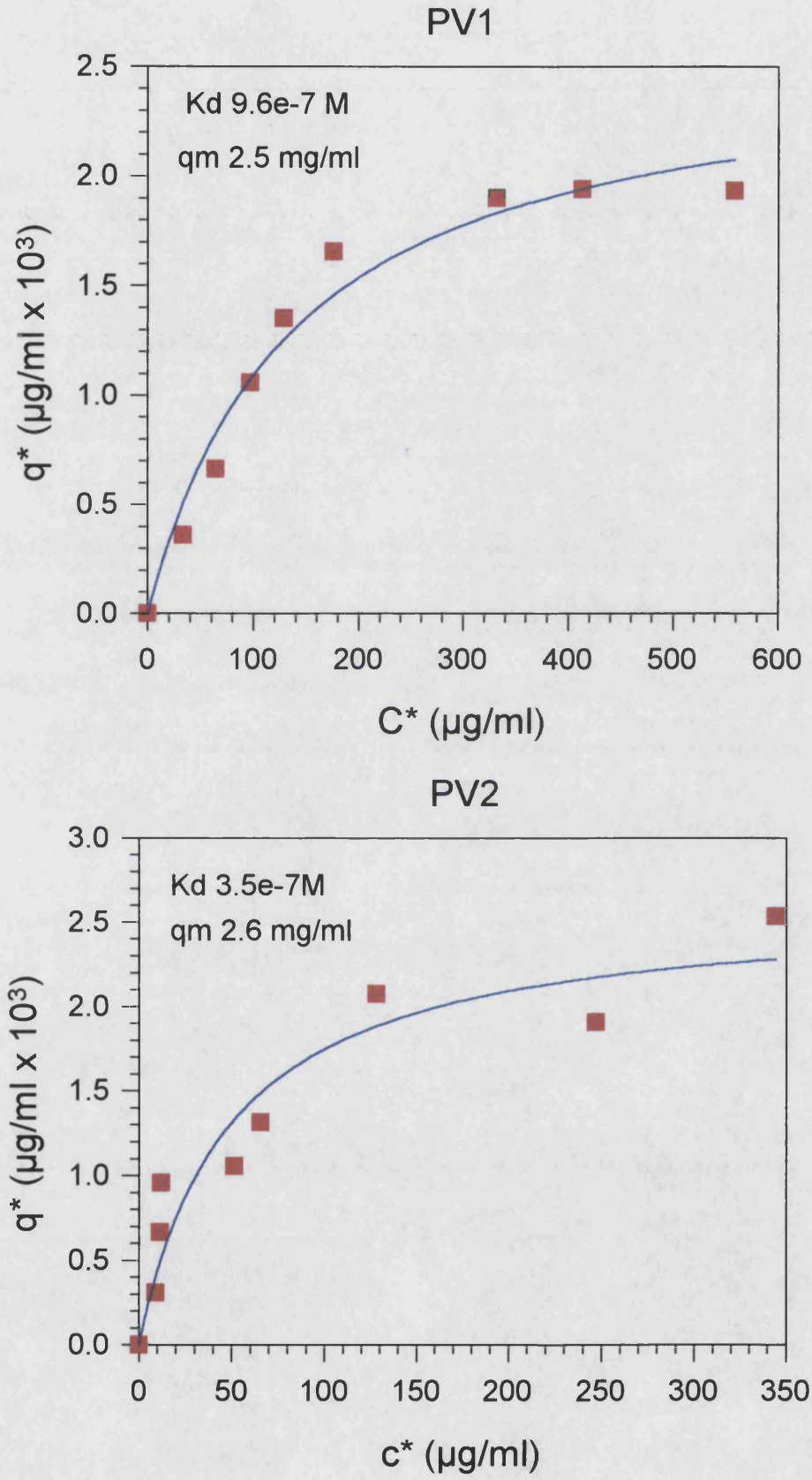


Figure 4.5: *Equilibrium adsorption isotherms of binding of LDH to PV1 and PV2 (Immobilised dye concentration 0.73 µmole ml^{-1} and 1.2 µmole ml^{-1} respectively). Lines indicate least squares fit to equation 4.5.*

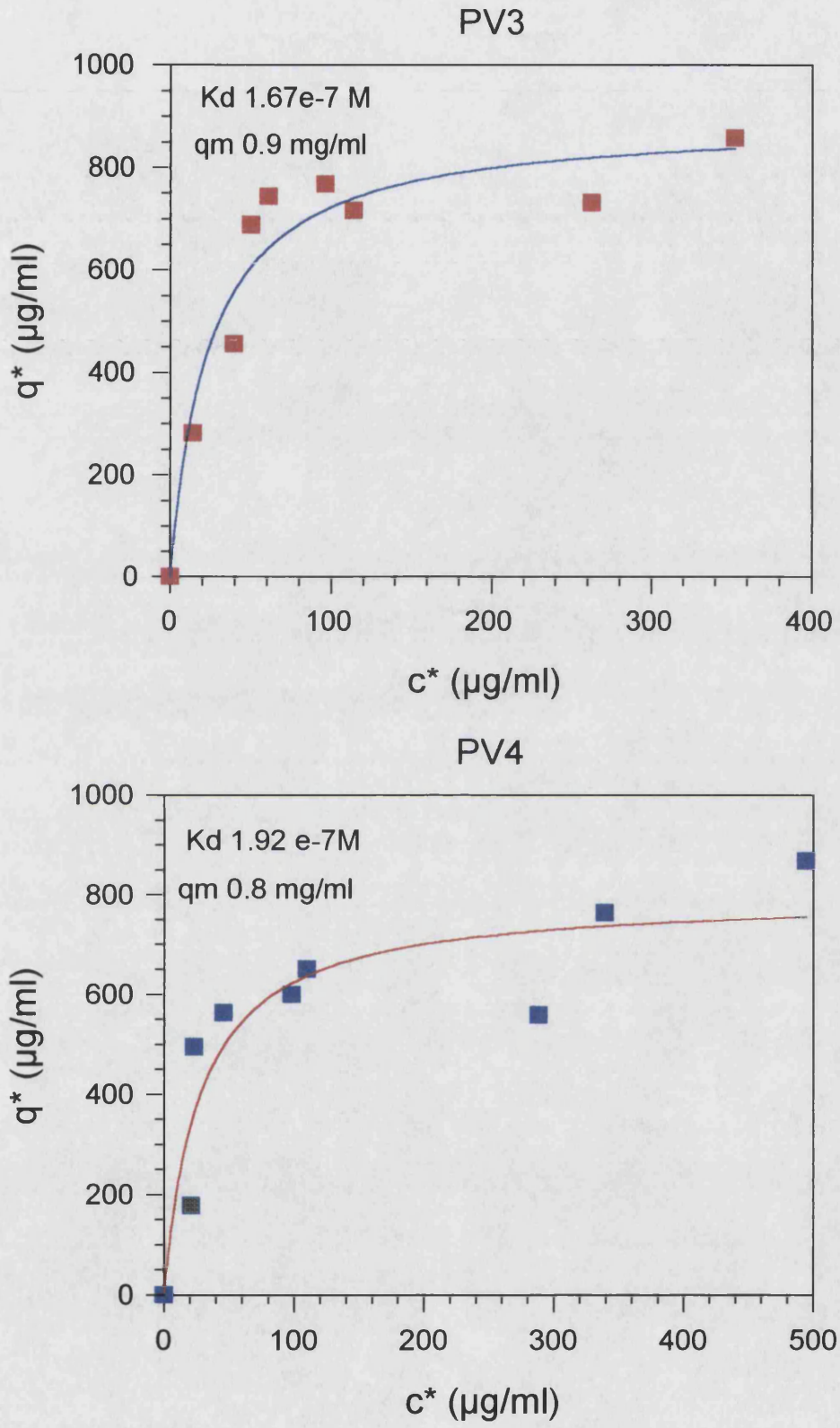


Figure 4.6: *Equilibrium adsorption isotherms of binding of LDH to PV3 and PV4 (Immobilised dye concentration 6.2 µmol ml^{-1} and 7 µmol ml^{-1} respectively). Lines indicate least squares fit to equation 4.5.*

However the maximum binding capacity (q_m) decreases with increasing dye loading (table 4.3). This is a reversal of the trend found with Blue Sepharose, where an increase in dye loading led to an increase in q_m (Boyer and Hsu 1992). Usually an increase in immobilised ligand tends to increase an adsorbent's binding capacity, because there is more ligand available to interact with protein in the mobile phase. The increase in capacity can also be explained by equation 4.1, where an increase in ligand (L) will push the equilibrium further to the right increasing P.L complex formation.

Table 4.3: Summary of equilibrium data obtained for PVA-polystyrene

Blue dextran PVA-polystyrene (code)	Immobilised dye concentration (μ mole ml^{-1})	Binding capacity q_m, (mg ml^{-1})	Dissociation constant, k_d x 10^{-7} M
PV1	0.73	2.5	9.6
PV2	1.2	2.6	3.5
PV3	6.2	0.9	1.92
PV4	7	0.8	1.92

The tentacle support differs from porous supports. In the case of the tentacle support because there appears to be an optimum number of ligands on the polymer, where the ligands may be more evenly spaced for protein interaction. It is interesting to note that the more highly substituted supports PV3 and PV4 have a lower q_m than the lower loaded supports PV1 and PV2 (table 4.3). This could be explained by the increasing number of dye molecules, which has the effect of increasing hydrophobic dye-dye interactions as described by Mayes (1993) and Maytum (1997). The increased dye-dye interactions reduce the dye availability and lowers the effective capacity of the adsorbent. Conversely the decrease in dye loading is leading to an increase in q_m which is probably due to there being greater dye availability with the reduction in dye-dye interactions.

4.3.5 Batch adsorption kinetics

The data obtained from the equilibrium experiments was useful to obtain estimates of K_d and q_m, but meaningful data for process design must be gained from kinetic experiments In order to understand how adsorption will occur if the process is scaled up.

The mutual depletion model (equation 4.4) cannot be used in its form because both c and q are time dependent variables. To allow integration, c can be eliminated by substitution of the mass balance equation for a well mixed vessel (equation 4.6) where $\alpha = v_{ads} / V_{tot}$. The equation now takes the form:

$$\frac{dq}{dt} = k_1(q_m - q)(c_0 - \alpha q) - k_2 q \qquad (4.7)$$

This equation can be integrated to allow calculation of q and c as a function of time using the boundary conditions t=0, $q=q^0$ and t=tq=q^t.

$$q_t = \frac{(b-\sqrt{x})(2k_1\alpha q^0 + b + \sqrt{x}) - (b+\sqrt{x})(2k_1\alpha q^0 + b + \sqrt{x})e^{(t\sqrt{x})}}{2k_1\alpha(e^{(t\sqrt{x})}(2k_1 q^0 + b - \sqrt{x}) - (2k_1\alpha q^0 + b + \sqrt{x})} \qquad (4.8)$$

where $x = (b^2 - 4k_1^2 c_0\, q_m)$ and $b = -(k_1 c_0 + k_1 \alpha q_m + k_2)$

Equation 4.8 was used as a model to determine the value the constants, where the values of c_o and α are known. Initial estimates of q_m, and K_d obtained from the equilibrium data (figure 4.5 and 4.6) were used as parameters for the model. The kinetic data are displayed in tables 4.4-4.7.

Table 4.4: Results for PV1 (Immobilised dye concentration 0.73 μmole ml^{-1})

Initial protein concentration C_o (μg ml^{-1})	Maximum binding capacity q_m (mg ml^{-1})	Dissociation constant K_d (mol l^{-1})	Forward rate constant k_1 (l mol^{-1} s^{-1})
33	16.41	6.2×10^{-7}	0.012
63.81	14.95	1.1×10^{-6}	0.012
96.8	8.09	9.2×10^{-7}	0.024
128.44	1.06	9.4×10^{-7}	0.013
175.62	7.01	1.2×10^{-6}	0.031
331.51	3.50	1.1×10^{-6}	0.025
431.25	2.76	9.6×10^{-7}	0.031
558.51	2.57	9.6×10^{-7}	0.017

Table 4.5: Results for PV2 (Immobilised dye concentration 1.2 μmole ml^{-1})

Initial protein concentration C_o (μg ml^{-1})	Maximum binding capacity q_m (mg ml^{-1})	Dissociation constant K_d (mol l^{-1})	Forward rate constant k_1 (l mol^{-1} s^{-1})
32.4	2.63	4.7×10^{-7}	0.034
64.51	3.14	3.3×10^{-7}	0.038
96.31	3.75	3.1×10^{-7}	0.031
133.97	2.17	3.6×10^{-7}	0.055
159.10	2.58	3.8×10^{-7}	0.031
288.71	2.60	3.5×10^{-7}	0.021
397.85	2.41	6.3×10^{-7}	0.014
566.11	5.51	3.2×10^{-6}	0.046

Table 4.6: Results for PV3 (Immobilised dye concentration 6.2 μmole ml^{-1})

Initial protein concentration C_o (μg ml^{-1})	Maximum binding capacity q_m (mg ml^{-1})	Dissociation constant K_d (mol l^{-1})	Forward rate constant k_1 (l mol^{-1} s^{-1})
37.22	0.76	1.9×10^{-7}	0.13
76.17	0.75	1.9×10^{-7}	0.11
103.67	0.95	1.6×10^{-7}	0.12
122.58	0.92	1.6×10^{-7}	0.14
151.62	0.89	1.6×10^{-7}	0.12
170.02	0.84	1.8×10^{-7}	0.079
322.04	0.78	2×10^{-7}	0.05
477.65	0.88	1.7×10^{-7}	0.049

Table 4.7: Results for PV4 (Immobilised dye concentration 7 μmole ml^{-1})

Initial protein concentration C_o (μg ml^{-1})	Maximum binding capacity q_m (mg ml^{-1})	Dissociation constant K_d (mol l^{-1})	Forward rate constant k_1 (l mol^{-1} s^{-1})
36.31	0.39	2×10^{-7}	0.134
70.05	1.04	2×10^{-7}	0.137
93.85	0.87	1.9×10^{-7}	0.126
144.02	0.75	1.9×10^{-7}	0.112
160.72	0.71	1.9×10^{-7}	0.108
332.17	0.66	2.3×10^{-7}	0.042
400.72	0.83	1.9×10^{-7}	0.048
558.24	0.89	1.9×10^{-7}	0.048

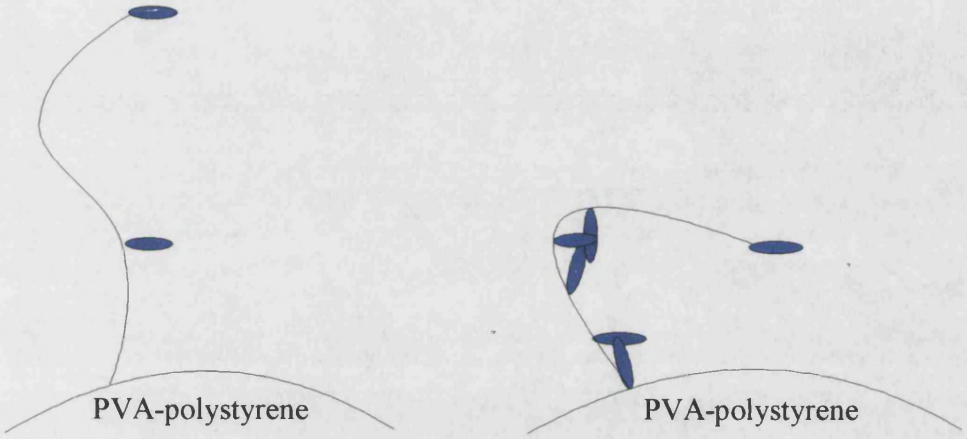
From the data (table 4.4-4.7) the forward rate constant (k_1) is the same order of magnitude for PV1 and PV2. However, the values of k_1 for PV3 and PV4 are an order of magnitude higher than the values obtained for PV1 and PV2. This leads to the tentative suggestion that the dye loading is affecting the rate of protein adsorption. The higher dye loading decreases the number of ligands available for binding, thus bulk transfer of the protein maybe more rapid for PV3 and PV4.

The dissociation constant (K_d) generally has a value of 10^{-7} M irrespective of dye loading. The values are in good agreement with the K_d values obtained from the equilibrium data (table 4.3). This shows the binding between LDH and Cibacron blue is a high affinity binding interaction. The values of K_d for the tentacle support are of the same order of magnitude for K_d obtained for blue Sepharose CL-6B binding to LDH (Liu and Stellwagen, 1987). This high affinity interaction is expected because the dye is quite a potent competitive inhibitor of LDH (K_i 0.13 μM; Thompson and Stellwagen, 1976), because the dye competes with NADH for the cofactor binding site.

The binding capacity (q_m) displays a lot of variation with the initial protein concentration (table 4.4-4.7). The largest variation was shown by PV1, the data for this support was susceptible to the largest errors using the integrated rate equation model (equation 4.8). The reason for this is unclear, it could that any slight deviation of the model from the raw data may lead to greater errors when the q_m is calculated. Although, this variation in q_m is repeated throughout the series PV2-PV4, the errors associated with q_m are less. This is displayed by more uniform values of q_m which are similar to the values obtained from the equilibrium data (table 4.3).

The increase in dye loading leads to a decrease in q_m with the higher loaded supports PV3 and PV4, having q_m less than 1 mg ml^{-1}. This could be due to the proximity of neighbouring dye molecules being such that there is an increase in intramolecular hydrophobic interactions. This may cause the dye-dextran polymers to coil into a tighter compact configuration (Mayes, 1992). Hence reducing the availability of dye ligands for protein binding (figure 4.7). The lower loaded supports PV1 and PV2 have higher q_m

values. This may be due to a reduction in intramolecular dye-dye interactions. The dye-polymer would most probably be in an extended conformation, which exposes a larger number of dye ligands to the bulk solution, made available for binding.



low loaded polymer chain higher loaded polymer chain

Figure 4.7: *Schematic diagram of the effect of dye loading on the dextran polymer*

A similar trend was found using porous blue Sepharose (Labru and Clonis, 1995). In the case of the porous support, the decrease in ligand concentration led to more dye molecules interacting in protein binding, because a smaller fraction of dye molecules where sterically hindered.

Another possible explanation for the variation in values of q_m and k_1 was the model (equation 4.8) was unable to fit the data correctly (figure 4.8). At lower initial protein concentrations the model was able to fit the data closely with only a slight deviation from the data. However, as the protein concentration increases, equilibrium is harder to determine at higher concentrations due to a slow continuos adsorption rate after 250 seconds. This phenomenon is also displayed by blue dextran in free solution, that there is a high affinity binding population at low protein concentrations and a lower affinity site at lower concentrations (Mayes *et al.*, 1990). The model is unable to adequately fit the data, thus causing a greater amount of deviation from the experimental data (figure 4.8). This phenomenon was displayed by all the supports regardless of dye loading. It appears that this deviation from the data maybe responsible for the inconsistent values obtained

for q_m and k_1 (table 4.1-4.2). The values of q_m and k_1 for PV1 and PV2 in particular, were more susceptible to this deviation, whilst the values of q_m and k_1 for higher loaded supports (PV3 and PV4) are not as sensitive to the deviation. The model is not robust enough to describe the data fully, a more complex model is required.

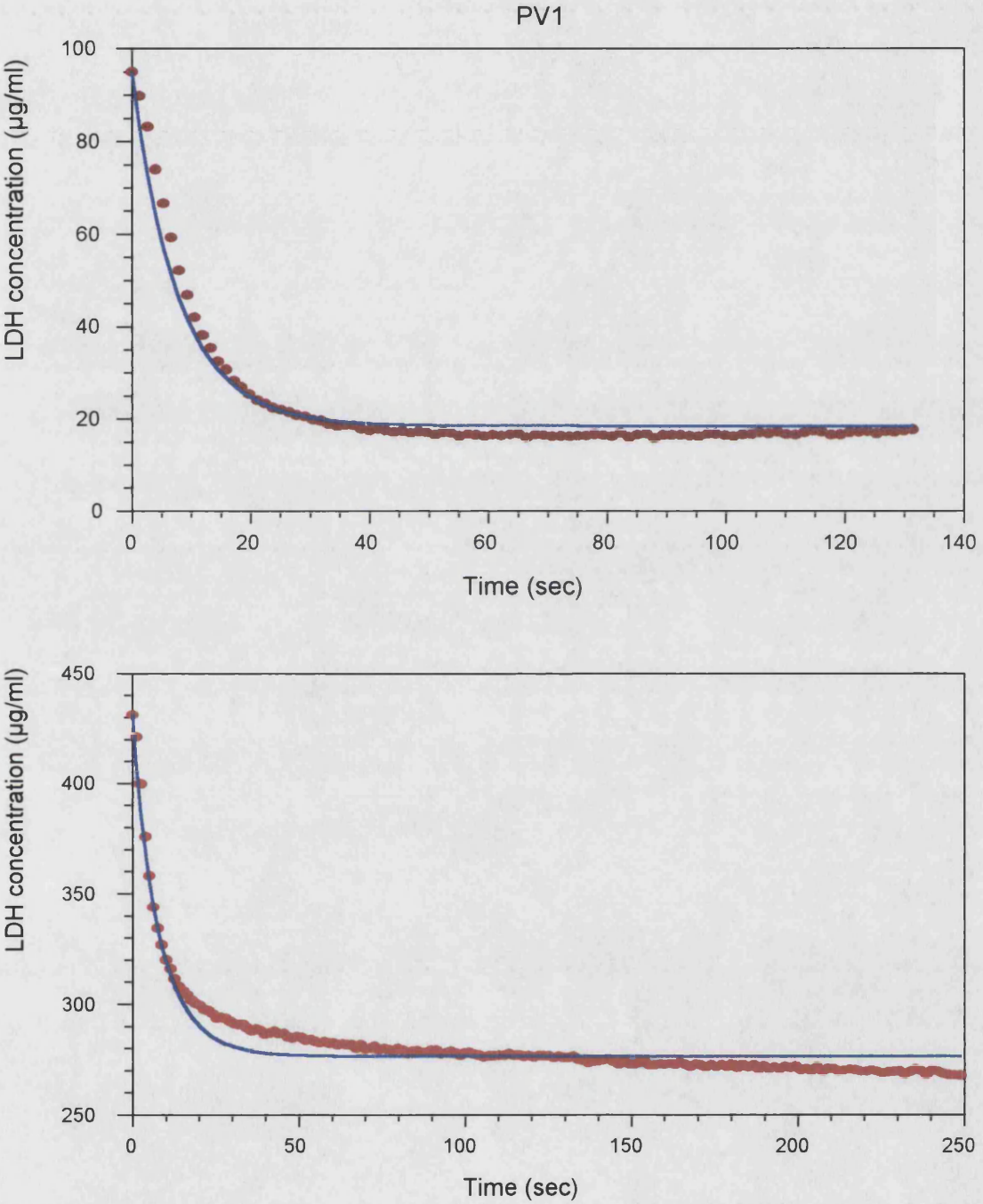


Figure 4.8: *Adsorption kinetics of LDH onto PV1 (immobilised dye concentration 0.73 µmole ml^{-1}).*

Kinetic protein adsorption kinetic experiments were carried out as described by Horstmann *et al.*, (1986) The figure shows the fit of equation 4.7 (blue) to the experimental data (red). The initial LDH concentration was 95 µg ml^{-1} and 430 µg ml^{-1} respectively.

Analysis of the bead surface using a scanning electron microscope shows some interesting characteristics. The surface is not very smooth, even after the coating with polyvinyl alcohol (figure 4.9), the blue dextran however appears to be immobilised as small clumps on the surface of the support (figure 4.10).

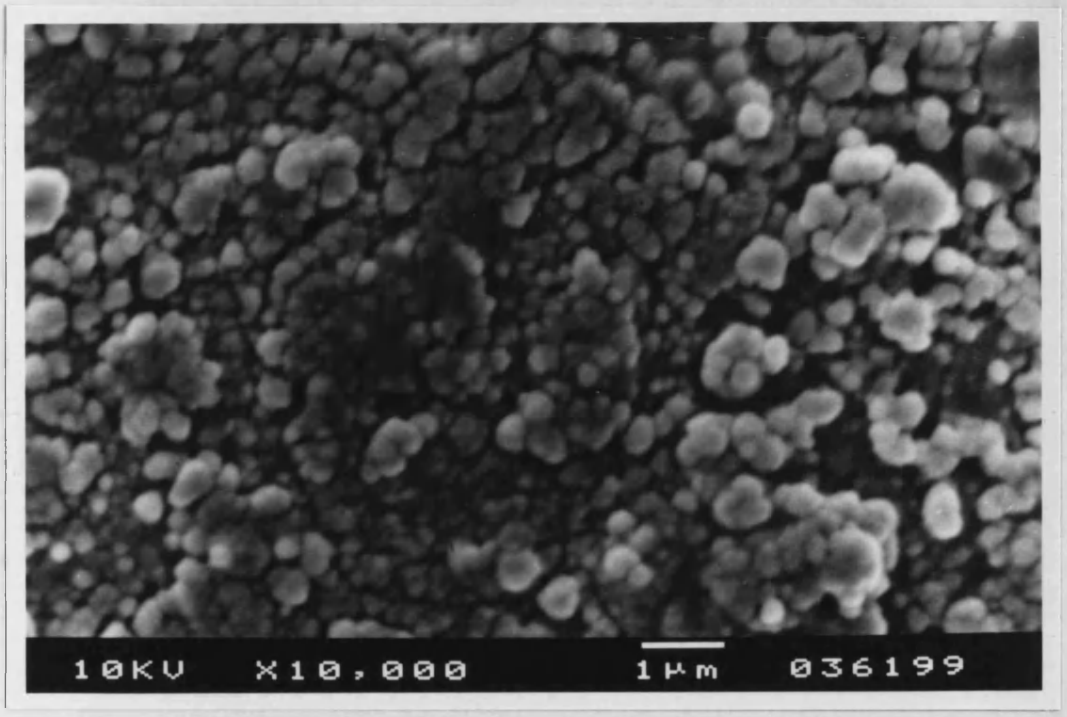


Figure 4.9: *Electron micrograph of the surface of PVA-polystyrene (mag x 10,000).*

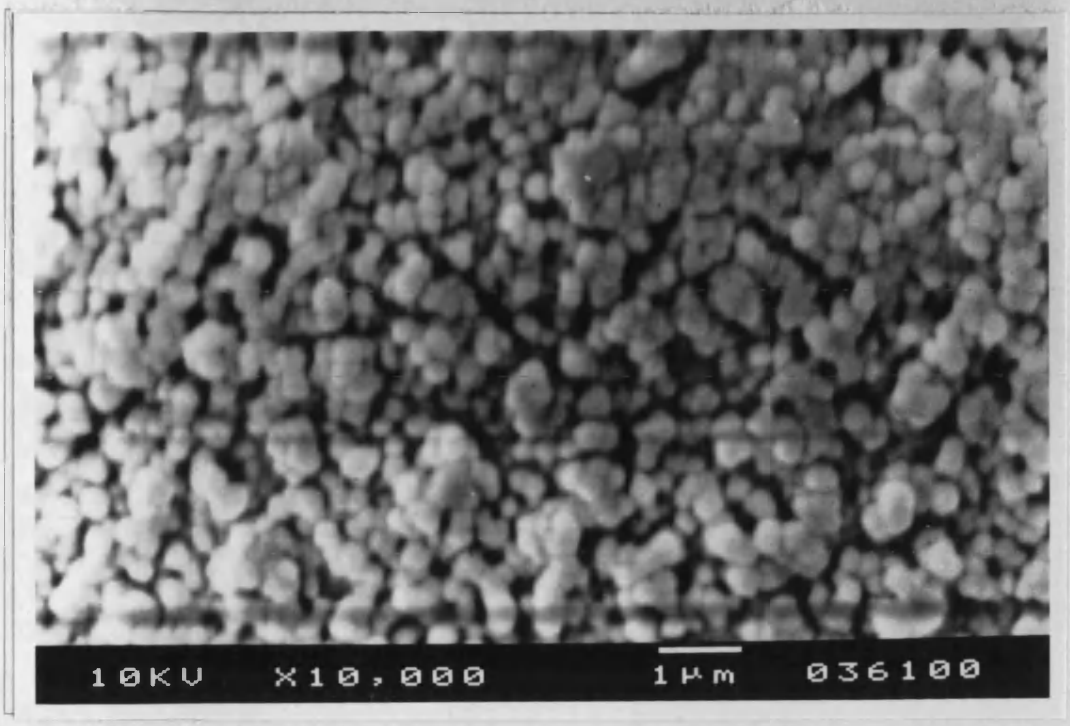


Figure 4.10: *Electron micrograph of the surface of blue dextran PVA-polystyrene (mag x 10,000).*

Ligand utilisation is the ratio of the maximum binding capacity (q_m) to ligand concentration (maximum theoretical capacity) assuming a monovalent interaction; the ligand utilisation for the tentacle supports PV1-PV4 is 6.9%, 1.8%, 0.1%, and 0.1% respectively. In comparing ligand utilisation of porous blue Sepharose CL-6B with similar dye loadings are approximately 2% for yeast alcohol dehydrogenase (tetramer 148 kD, Boyer and Hsu, 1992). The lower loaded tentacle supports (PV1 and PV2) compare most favourably especially PV1 which has a relatively low dye substitution (0.7 μmole ml^{-1}) but a high q_m. The importance of this finding is that there appears to be an optimum dye loading for the tentacle support, and that the support appears to perform better with lower ligand substitution. Despite the reasonable performance of the tentacle support the overall ligand utilisation is still low (<10%), this area has yet to be fully addressed in affinity chromatography. An excess of immobilised ligand is not being used and is thus wasted. This becomes significant especially if the ligand is expensive and the process is to be scaled up.

Although the protein binding capacities were not particularly high (section 4.3.5), the performance can be expressed as a ratio:

$$\frac{\text{actual capacity } (\text{mg ml}^{-1})}{\text{theoretical capacity } (\text{mg ml}^{-1})} \qquad (4.9)$$

The theoretical capacity is the maximum amount of protein which can bind to the total available surface area.

PVA-polystyrene has a specific surface area of 0.1 $m^2 g^{-1}$

$\Rightarrow$ 1g of PVA-polystyrene $\equiv$ 2 ml bed volume (section 2.3.5)

$\Rightarrow$ surface area per unit volume = 0.2 $m^2 ml^{-1}$

$\Rightarrow$ polystyrene capacity for LDH $\approx$ 0.5 mg ml^{-2} (Bangs)

Therefore, one ml of PVA-polystyrene can accommodate:

0.2 m^2 x 2.5 mg $m^{-2} \approx$ 0.5 mg

Table 4.8 shows a 14 fold increase in protein binding for the tentacle support (PV1). The tentacle supports (PV3 and PV4) with low q_m show approximately a 2 fold increase. Thus showing dramatically how the tentacle configuration of blue dextran PVA-polystyrene can enhance protein binding. These results are in agreement with the work of Muller (1990). Ionic monomers grafted on the support surface in a tentacle conformation, dramatically increased the protein binding capacity when compared to the native support.

Table 4.8: Enhancement of protein binding capacity due to the immobilised tentacle polymer

Blue dextran PVA-polystyrene	Protein binding capacity (mg ml^{-1})	Actual Capacity / Theoretical Capacity
PV1	7	14
PV2	1.2	2.4
PV3	0.9	1.8
PV4	0.8	1.6

4.3.6. Pressure flowrate characteristics of blue dextran PVA polystyrene and blue Sepharose CL-4B

The tentacle support shows a linear relationship between back pressure and linear flowrate (figure 4.11), and displays the characteristics of a "rigid" bead (Stewart *et al.*, 1992). The rigid nature of the bead stems from the fact that there is a high proportion of divinylbenzene present in the support. The higher cross linked polystyrenes are more resilient to chemical attack and are physically more stable (Ellingsen *et al.*, 1990). This suggests that the tentacle support is suitable for use in either high or intermediate pressure chromatography, which would be suitable for large process scale applications. A linear flowrate of 1200 cm h^{-1} produced a back pressure of only 0.06M Pa. This suggests that the tentacle support is suitable for use in either high or intermediate pressure chromatography, which would be suitable for large process scale applications.

In contrast for Sepharose CL-6B a linear flowrate of only 350 cm h^{-1} produced a back pressure of 0.2 MPa. Above this flowrate bed compression occurs resulting in a dramatic increase in back pressure. Thus exposes cross linked agaroses mechanical limitations which precludes the use of this support from intermediate and high pressure applications. Cycle times of washing, elution and regeneration are also greatly increased because of the inability of the support to withstand high flowrates.

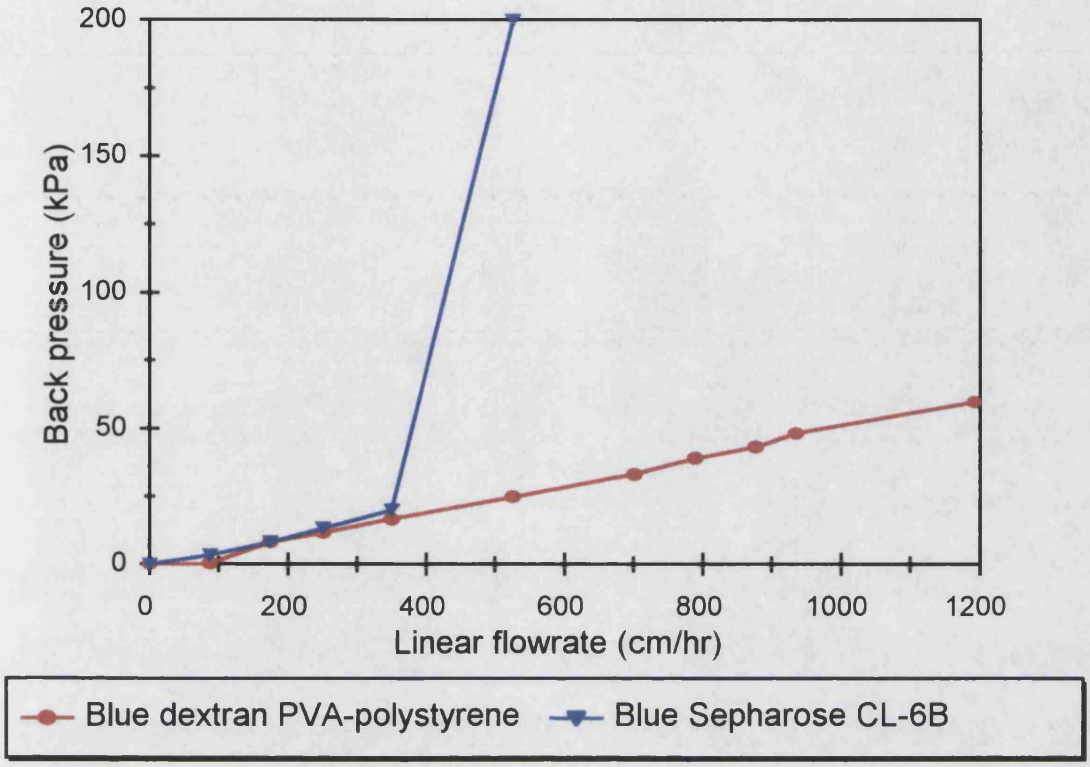


Figure 4.11: *Pressure flowrate characteristics of blue dextran PVA-polystyrene and blue-Sepharose CL-6B.*

Blue dextran PVA polystyrene (1 ml) was packed into a small column, and the bed was subjected to steadily increasing flow rates using a peristaltic pump. A pressure gauge was located at the inlet and the resulting increase in back pressure was measured. The experiment was repeated with blue Sepharose CL-6B using the same conditions.

4.3.7 Purification of fumarase

Fumarase is a tetrameric enzyme of molecular weight 198 kD (Beeckmans and Kanarek, 1977) and catalyses one of the reactions of the Tri-carboxylic acid cycle, where fumarate is converted to L-malate (figure 4.12).

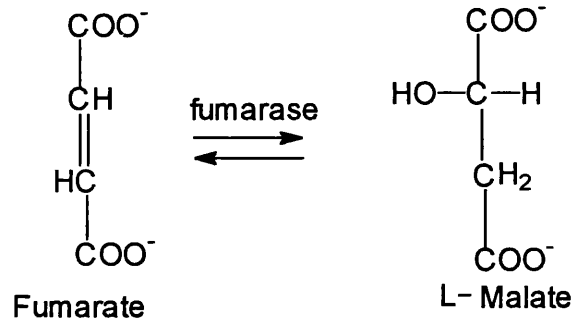


Figure 4.12: *Reaction catalysed by fumarase*

A crude sample of rabbit liver fumarase (40-60% ammonium sulphate cut) was kindly donated by Sigma Chemical Company. The purification of the enzyme in industry is a difficult and convoluted one. Immobilised pyromellitic acid is used as an affinity step, which reduces the amount of contaminating proteins. Further purification is carried out using blue-Sepharose.

It is assumed that blue-Sepharose has two binding sites for fumarase. A highly selective site for fumarase and a lower affinity site. BSA is used to block both of these sites. L-malate is used to displace BSA bound to the fumarase specific site. The lower affinity site will remain blocked by BSA, and the blue-Sepharose is divided into two portions. One portion of the blue-Sepharose is then washed to remove L-malate. The crude extract is then added to this portion and fumarase will bind. As the less specific site has been blocked by BSA. Contaminating proteins should not bind and can be washed off. Fumarase is eluted by the addition of L-malate in buffer.

The remaining portion of blue Sepharose is packed into a column and washed with L-malate containing buffer. The fumarase will not bind to the column in the presence of L-malate, because fumarase has a higher affinity for free L-malate. The fumarase will therefore pass straight through the column. Any remaining contaminants should again bind to the blue-Sepharose column. At this stage fumarase is now sufficiently pure.

It is thought that the problems in purification occur because of the ligand's inability to interact properly with the enzyme. Cibacron blue is attached directly to the matrix backbone of Sepharose. In the case of the tentacle support the dye ligands are attached to polymer molecules and this difference may simplify the purification procedure for fumarase.

The enzyme did not interact very strongly with the column of blue dextran PVA-polystyrene (PV1). PV1 was chosen because this support had the highest capacity of all the blue dextran PVA-polystyrene supports (section 4.3.5, tables 4.4-4.7). On several column runs, most of the enzyme appeared in the column breakthrough. The small amount which bound could be eluted biospecifically with malic acid. The eluted fractions contained fumarase activity but with low specific activity. The purification tables 4.8 and 4.9 summarise the data.

Table 4.8: *Isolation of fumarase from rabbit liver*

Step	**Volume ml**	**Enzyme activity U ml^{-1}**	**Protein conc mg ml^{-1}**	**Specific activity U mg^{-1}**	**Total activity IU**	**Yield %**
ammonium sulphate	6	0.25	5	0.05	1.5	100
column eluate	4	0.01	0.064	0.16	0.0365	2.7

Table 4.9: Isolation of fumarase from chicken heart

Step	Volume ml	Enzyme activity U ml^{-1}	Protein conc mg ml^{-1}	Specific activity IU mg^{-1}	Total activity U	Yield %
ammonium sulphate	17	0.36	6.68	0.054	6.14	100
column eluate	2.81	0.028	0.016	1.75	0.08	1.3

The elution volume was quite large which may explain the low activity of the activity of the enzyme. However, there is an increase in the specific activity of the enzyme. There was no increase in fumarase activity using higher concentrations of L-malate in the elution buffer. This shows that the majority of fumarase passes straight through the column without binding to the tentacle support. The fumarase that binds, appears to bind as a diffuse band on the column, hence the relatively large elution volumes.

The column eluate was analysed by SDS page electrophoresis which showed that there was a band at approximately 39 kD (figure 4.13). A closer inspection of the gel revealed some very faint bands of high molecular weight. The low concentration of purified protein (table 4.4 and 4.5) necessitated the need for concentration with PEG. This step yielded protein bands of molecular weights; 95 000, 60 000, 54 000, 50 000 (Fumarase) and 36 000 respectively (figure 4.14). The band of 50,000 kD corresponds closely with the size of the fumarase purified by Beeckmans and Kanarek (1977).

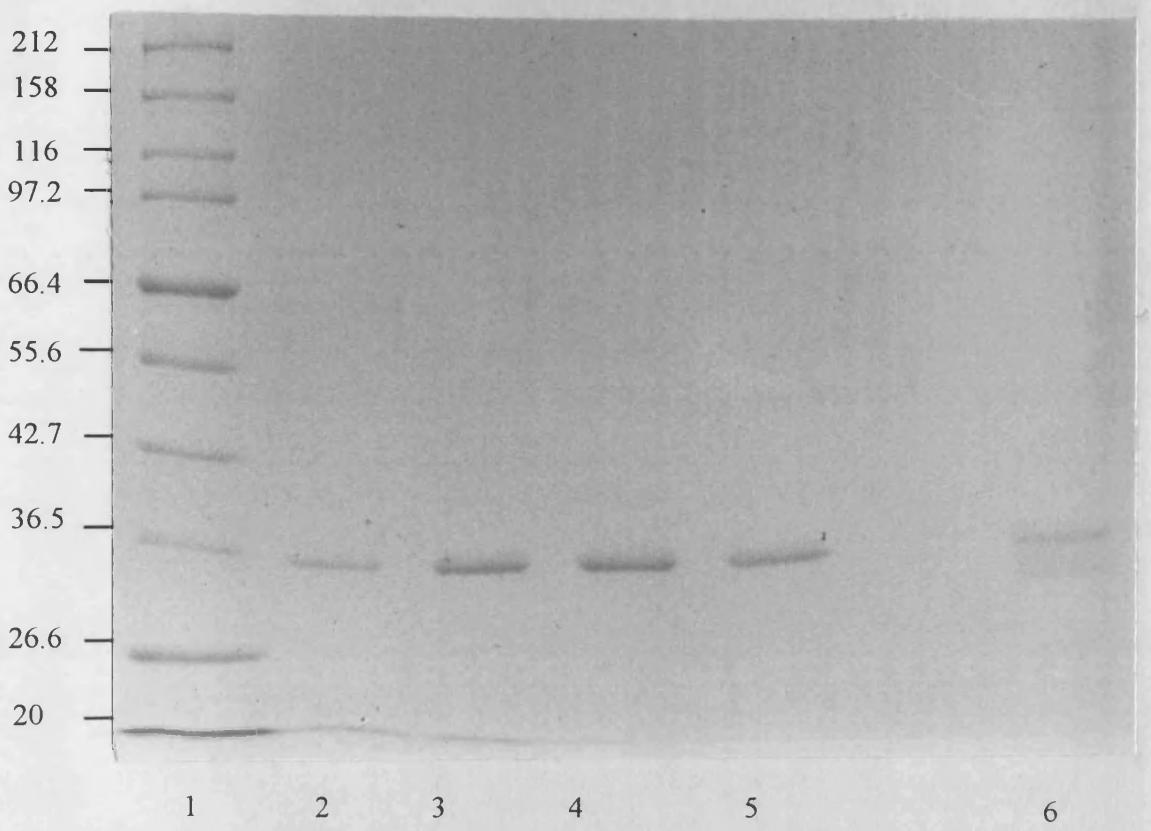


Figure 4.13: *SDS PAGE*

Lanes: (1) molecular weight markers (Sizes in kD on left), (2-4) L-malate eluted protein from blue dextran PVA-polystyrene (approximately 1,2,4 and 6μg protein respectively), (6) L-malate eluted protein from blue dextran PVA-polystyrene (approximately 2 μg protein) in absence of mercaptoethanol.

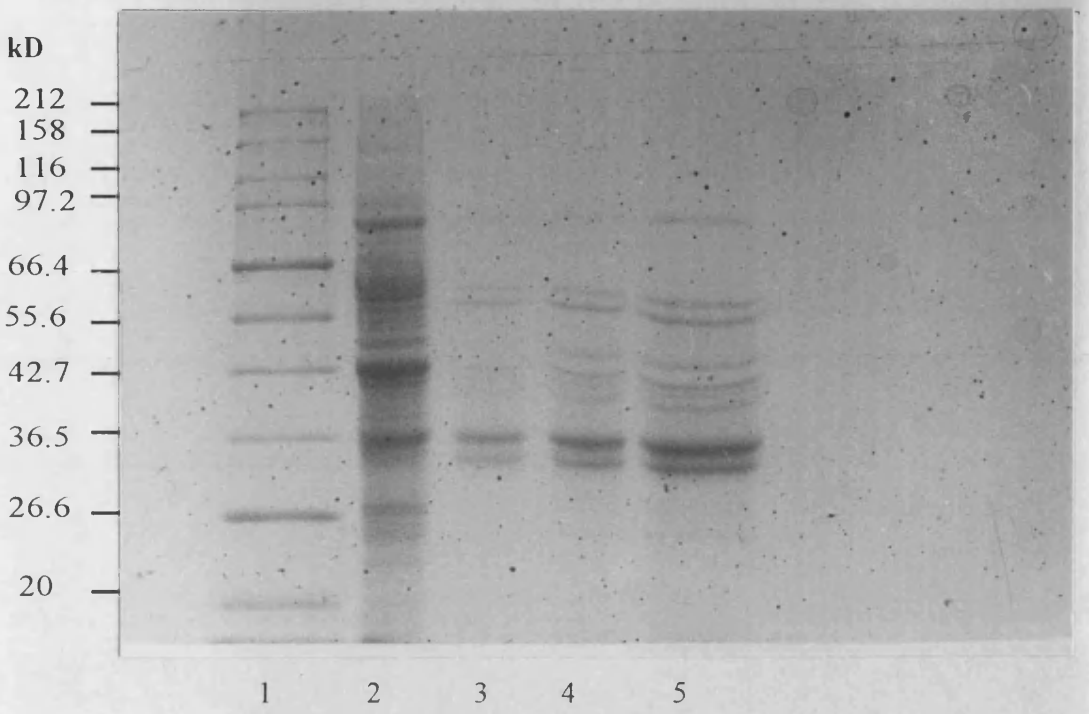


Figure 4.14: *SDS-PAGE*

Lanes: (1) molecular weight markers (sizes in kD on left), (2) crude ammonium sulphate fraction (60-80% cut), (3-5) PEG concentrated L-malate eluted protein from blue dextran PVA-polystyrene (5, 10 and 20 μg protein respectively) in absence of mercaptoethanol.

Fumarase isolated from both pig heart and liver (Beeckmans and Kanarek, 1977) using pyromellitic acid as an affinity step also produced fumarase with several contaminating proteins. The lack of purity from the affinity steps could be due to the weak cationic exchange character of the ligands Cibacron blue and pyromellitic acid, which would promote non-specific binding (figure 4.15). To obtain fumarase of high purity Beeckmans and Kanarek (1977) crystallised the crude eluate from the pyromellitic acid affinity column by dialysis against increasing concentrations of ammonium sulphate.

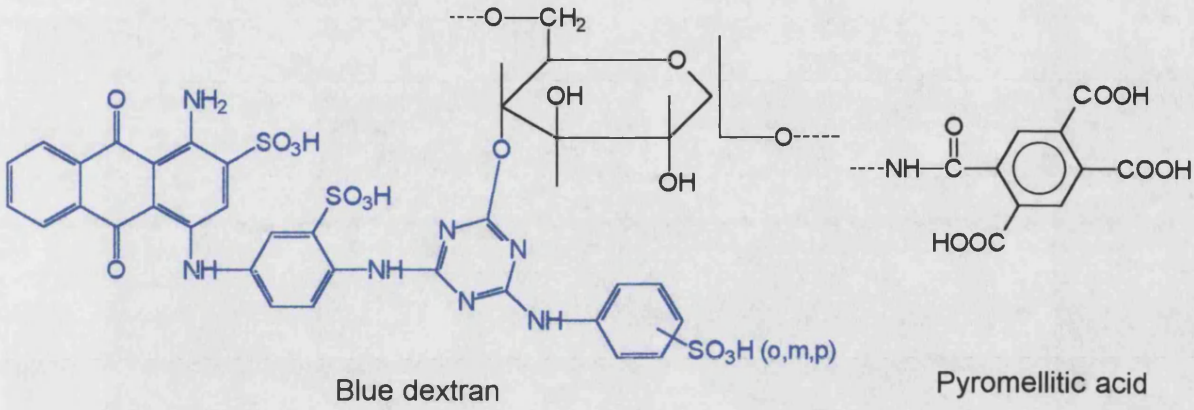


Figure 4.15: *Partial structures of immobilised blue dextran and pyromellitic acid*

Thus the binding between fumarase and Cibacron blue attached to polymer chains does not appear to be enhanced which was disappointing. It was thought that there might be steric problems preventing fumarase from interacting with the dye ligand attached directly to Sepharose backbone (Plant, 1996).

4.4. CONCLUSIONS

The PVA-polystyrene beads were was coated with dextran using epoxide activation to immobilise the polymer. The support was derivatised with Cibacron blue; the highest immobilised dye concentration was 7 μmole ml^{-1}. Higher loadings were not attempted although this would be feasible, because the immobilised blue dextran has similar characteristics to the soluble polymer. Cibacron blue has been immobilised up to a dye loading of 300 mol dye:per mol of dextran. The blue dextran PVA-polystyrene is stable without noticeable dye leaching. Also the acid hydrolysis required several incubations to liberate all the immobilised dye.

The results show that the theory of the tentacle support is a reality because the tentacle support reaches equilibrium with protein five times faster than porous blue Sepharose CL-6B (Gyepi-Garbrah *et al.*, 1996). The underivitised dextran PVA-polystyrene support also displayed low non-specific binding which is very important for an affinity matrix. High non-specific binding of the matrix will ultimately decrease the purity of the final product. Thus all the observed protein binding is due to the immobilised ligand and the eluted target molecule should be of high purity.

Analysis of the equilibrium data for binding of the tentacle support to LDH revealed that the dissociation constant (K_d) decreased with increasing dye loading. This is also found with free blue dextran in solution and with blue Sepharose. The dissociation constant was to the order of 10^{-7} M which is a high affinity binding interaction. The capacity of the tentacle support decreased with increasing dye loading which is the opposite with porous supports such as blue Sepharose. This could be due to there being an optimum dye loading above which there is an increase in intramolecular dye-dye stacking interactions. This leads to a decrease in the hydrodynamic size of the molecule and reduces the amount of ligand available for binding.

Analysis of the kinetic data gave similar results to the equilibrium data, the K_d was of the order of 10^{-7} M. The value for the K_d was relatively constant irrespective of dye loading. The capacity of the supports also showed the same trend of the capacity decreasing with increasing dye loading, but markedly there was a dramatic decrease in capacity with the two highest dye loadings. The reasons for this reduction in capacity is most likely to be

due to the number of intramolecular dye-dye interactions increasing and reducing the availability of the dye molecules. The ligand utilisation was however disappointing. The lowest dye loading had a utilisation of approximately 7%. The other loadings were 0.1% which was particularly low when compared to blue Sepharose which displayed a ligand utilisation of approximately 2%, for an enzyme of similar molecular weight. The lowest loading (PV1) displayed a respectable ligand utilisation because most porous adsorbents rarely have ligand utilisation values above 3%. Thus for the tentacle support the low ligand loading means that there are less problems of ligand leakage, This fact is especially important in the case of therapeutic proteins where the clearance has to in the range of pmoles (10^{-15}) of contaminants. The tentacle configuration increases the "effective surface area" of PVA-polystyrene, resulting in a 14 fold increase in protein binding capacity. However, it is unknown how far the blue dextran polymers tentacles extend out from the support surface. Electron micrographs of the tentacle support surface showed that the dextran polymer appeared to immobilised as clumps. Thus the tentacle formation could be similar to loose loops extending out from the bead surface.

The hydrodynamic behaviour of the tentacle support displayed the characteristics of a "rigid" bead. In contrast blue Sepharose is a soft bead and compression occurred at relatively low linear flowrates. Thus the tentacle support could be used in intermediate to high pressure chromatography, which also means that cycle times of adsorption, elution and regeneration can be greatly reduced, without deleterious affects to the support. The flow characteristics of the tentacle support appear similar to the that of the Poros® support described by Aefyan *et al.*, (1990a). This is to be expected because Poros® is also a co-polymer of styrene and divinyl benzene, the only difference in the bead structures is the non-porous nature of the tentacle support. Another significant fact is that the presence of the polymer on the support surface does not form a soft compressible layer. If this was the case, there would have been a gradual increase in back pressure as the soft polymer layers compressed against each other reducing the inter particle spaces for fluid flow.

The purification of fumarase was not successful. The enzyme did not interact strongly with the tentacle support; most of the enzyme passed through the column unbound. The

"purified" fumarase could only be visualised on SDS-PAGE after concentration with PEG. However, there was an increase in specific activity despite the low purity. In hindsight, perhaps a crude enzyme requiring an adenyl co-factor would probably have been a better choice of protein to purify. The interactions between the dye and such proteins have been well documented in the literature.

CHAPTER FIVE

MONOMER AND POLYMER SYNTHESIS

5.1. INTRODUCTION

Although blue dextran PVA-polystyrene (chapter four) had alleviated the problem of adequate polymer immobilisation. A method was still required to immobilise a sufficient amount (>1 mg ml^{-1}) of pre-characterised polymer on to the surface of PVA polystyrene. As there are a few commercially available polymers which have hydroxyl side chains as illustrated in figure 5.1, the solution appears to be polymer synthesis.

There are two alternatives;

i) Polymerise alkali stable monomers which have primary hydroxyl side chains;

ii) Synthesise an alkali stable monomer which contains several primary hydroxyl groups and polymerise this monomer.

Alkaline stability is necessary because the coupling reactions of dye reaction and the immobilisation of the subsequent dye polymer requires an alkaline pH.

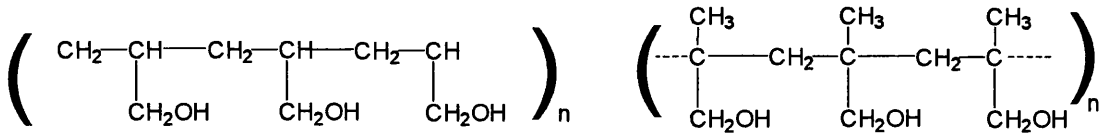


Figure 5.1: *Structures of suitable synthetic hydrophilic polymers*

The pre-characterised polymer can be synthesised in two different ways:

a) Synthesis of homopolymers and co-polymers using the monomers shown in figure 5.2. Subsequently any polymers produced will be reacted with dye thus forming a tentacle polymer for immobilisation.

b) Synthesise a monomer of the dye (Mazza *et al.*, 1989) and co-polymerise the dye monomer with a hydrophilic monomer. Thus synthesising another polymer which can be immobilised as a tentacle polymer. This tentacle co-polymer would be more desirable. The primary hydroxyl groups would then be free to react with epoxy groups on the surface of PVA-polystyrene. This may help in increase the amount of polymer immobilised.

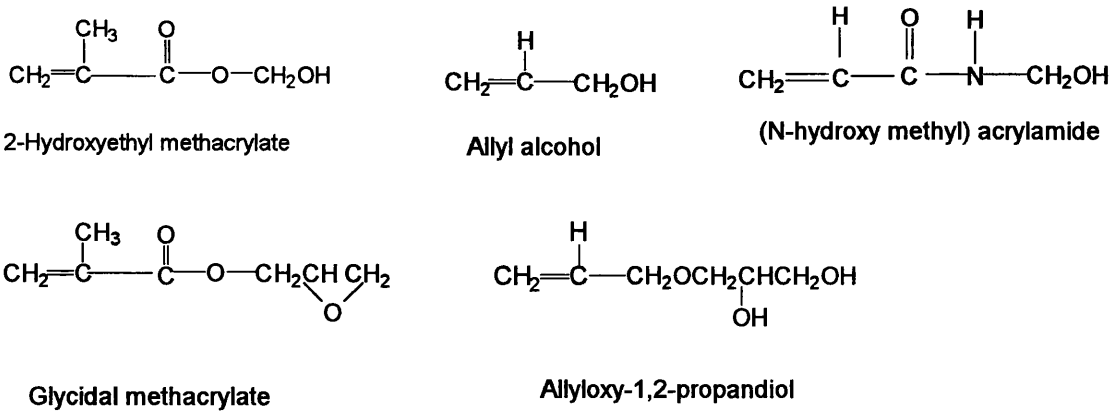


Figure 5.2: *Hydrophilic monomers suitable for production of synthetic hydrophilic polymers*

5.2. MONOMER SYNTHESIS

5.2.1a. Synthesis of N-[Tris(hydroxymethyl) methyl]methacrylamide

Tris(hydroxymethyl)aminomethane (Tris 31g, 0.255 mol) was added to 400 ml of anhydrous methanol in a three necked round bottomed flask (1 L) and heated under reflux until dissolved. Methacrylic anhydride (19 ml, 0.127 mol) was added dropwise and the reaction was left for four hours under reflux. This was then allowed to cool to room temperature. The reaction mixture was filtered to remove salt formed during the reaction. The reaction mixture was then placed on a rotary evaporator and the solvent was removed. A pale pink oily residue was left in the flask.

The oil was placed onto a silica column (280 g) which had been slurried in chloroform, and the products were eluted with 150 ml each of methanol/chloroform (1:1, (v/v)), methanol/chloroform (3:1, (v/v)) and finally methanol, fractions (20 ml) were collected. Fractions were analysed with TLC plates using methanol/chloroform (1:1, (v/v)) as solvent and the spots were stained with iodine.

The fractions containing the monomer were pooled and then solvent was removed by rotary evaporation. The resulting oil was triturated with diethyl ether. The ether was decanted into another flask. which was removed by rotary evaporation. The molecular weight was determined using mass spectroscopy.

5.2.2b. Mixed bed ion exchange purification of monomer

The reaction was carried out as described above, with the exception that after rotary evaporation of the reaction mixture, the resulting oil was dissolved in a minimum volume of 50% (v/v) methanol/water. The pH was raised to 9 using ammonium hydroxide, and loaded onto an anion exchanger column (100 ml, in OH^- form). The eluent was collected and neutralised immediately. and was reduced to a quarter of the original volume by rotary evaporation, then dissolved in a minimum of 50% (v/v) methanol/water. The pH was adjusted to 3.5 and loaded on to a cation exchange column (80 ml, in H^+ form). The eluent was immediately adjusted to pH 7 and the solvent was removed by rotary evaporation.

5.2.3. Dicyclohexyl carbodiimide mediated coupling of Tris and methacrylic acid

Tris (1 g, 8.25 mmol) was placed in 20 ml of DMF/pyridine (1:1, v/v) and heated to 70° C with stirring in a round bottom flask. When the Tris had dissolved, 233 µl (2.8 mmol) of methacrylic acid was added followed by 0.56 g (2.7 mmol) of dicyclohexyl carbodiimide (DCC). The reaction was allowed to continue for 3 hours, and allowed to cool to room temperature. Unreacted Tris was removed from the solvent by filtration and the filtrate was cooled to 0° C. Water was slowly added to the filtrate until a thick permanent white precipitate of O-dicyclohexyl urea had formed. The resulting suspension was filtered to remove the O-dicyclohexyl urea, and the solvent removed by rotary evaporation. The residue was triturated with pet ether (80-100° C fraction) and any remaining solvent was removed by rotary evaporation.

5.2.4. Monomer synthesis via saponification

Tris (2 g, 16.5 mmol) was placed in 35 ml of dry acetonitrile and 6.6 ml of dry pyridine in a three necked round bottom flask (150 ml) and methacryloyl chloride (8 ml, 76 mmole) was added dropwise. The mixture was refluxed under nitrogen for 4 hours. The reaction was allowed to cool to room temperature. The solvent was removed by rotary evaporation and 10 ml of KOH (5% (w/v)) in methanol was added. The reaction mixture was refluxed at 65° C for 15 minutes. The solvent was removed by rotary evaporation. The residue was chromatographed on a silica column and eluted with

chloroform/methanol (1:1, (v/v)). The crude product was analysed on TLC using iodine crystals or o-toluidine spray as a stain.

5.3. POLYMER SYNTHESIS

5.3.1. Polymerisation of 2-hydroxyethyl methacrylate

2-Hydroxyethyl methacrylate (HEMA), was passed through an inhibitor removal column (MEHQ column, 20 ml) and the eluate was kept on ice. A round bottomed flask containing 50% (v/v) monomer/water solution was degassed by bubbling nitrogen through the solution for 30 minutes. Ammonium persulphate (APS, 1 ml, 0.5% w/v) was introduced via a syringe, the reaction was allowed to proceed for 1 hour.

The experiment was repeated with the following variations:

I. 10% (v/v) HEMA

II. 10% (v/v) HEMA* 0.1% (w/v) initiator and reaction temperature at 50°C

III. 10% (v/v) monomer (N-hydroxy methyl) acrylamide: HEMA*, mole ratio 2:1), 0.1% (w/v) initiator and reaction temperature at 50°C.

(* No inhibitor removal step)

5.3.2. Polymerisation of hydroxy monomers

Using the method described by Fuller and Bright (1977):

a) (N-hydroxy methyl)acrylamide (2.82 ml, 30 mmol) and 4.1 ml (30 mmol) of glycidyl methacryate was added to 76 ml of 33% (v/v) ethanol/water. APS (150 mg) and N,N,N',N'-tetramethylethylenediamine (TEMED, 100 µl) were added to initiate polymerisation; the reaction took place in a closed vessel for 16 hours at 25°C.

The extent of polymerisation was determined by adding a small sample of the reaction mixture (50 µl) to 3 ml of acetone in a test tube. If a precipitate formed immediately the reaction mixture was rotary evaporated down to half the original volume. The polymer was precipitated by pouring the reaction mixture into a large excess of acetone and centrifuged at 27,000 g at 4°C. The pellets were resuspended in 40 ml of water and the polymer was dialysed against a large excess of water and then freeze dried.

b) The experiment was repeated as described previously, except that 200 µl of TEMED was added.

c) The following co-polymers and homopolymers were synthesised:

- Glycidyl methacrylate (2 ml, 15 mmol) and allyl alcohol (1 ml, 15 mmol), in 76 ml of 33% (v/v) ethanol/water. APS (150 mg) and 100 µl of TEMED were added to initiate the reaction.
- (N-hydroxy methyl)acrylamide (2.8 ml, 15 mmol) and allyl alcohol (1 ml, 15 mmol) in 76 ml of water. APS (150 mg) and 100 µl of TEMED were added to initiate the reaction.
- 3-allyloxy-1,2, propandiol (1.9 ml, 15 mmol) in 100 ml of water. APS (95 mg) and 95 µl of TEMED were added to initiate the reaction.
- Allyl alcohol (1 ml, 15 mmol) in 33 ml of water. APS (150 mg) and 200 µl of TEMED were added to initiate the reaction.
- (N-hydroxy methyl)acrylamide (2.8, 15 mmol) in 76 ml of water. APS (250 mg) of and 200 µl of TEMED were added to initiate the reaction.

The extent of polymerisation was determined as described previously.

5.3.3. Coupling of Cibacron blue to (N-hydroxy methyl)acrylamide-allyl alcohol co-polymer.

The reaction was carried out as described in section 3.2.1.

5.3.4. Coupling of blue (N-hydroxy methyl)acrylamide-allyl alcohol co-polymer to epoxy PVA polystyrene.

The procedure was carried out as described in section 3.2.5 except that blue (N-hydroxy methyl)acrylamide-allyl alcohol co-polymer was in the coupling buffer.

5.3.5. Stirred batch equilibrium experiments

Adsorption experiments were carried out as described in section 3.3.7.

5.4. RESULTS AND DISCUSSION

5.4.1. Monomer synthesis

The attempted synthesis of N-[Tris(hydroxymethyl)methyl]methacrylamide proved to be very difficult. The method of producing N-substituted hydroxy acrylamides or methacrylamides was first carried out by Paprotny and Jedlinski (1966). They reacted amino alcohols with acryloyl or methacryloyl chloride at room temperature. However, the amino alcohol (Tris) was insoluble under the conditions described by Paprotny and Jedlinksi (1966), and only just soluble in boiling acetonitrile. There appears to be no explanation for this insolubility which was surprising since these researchers used Tris to produce acrylamide and methacrylamide monomers. In an attempt to circumvent this problem a variety of solvents were tested to dissolve Tris; DMF, 1,4-dioxane, THF and methanol. Tris was only soluble in boiling methanol. Methacrylic anhydride was used instead of the more reactive acid chlorides because the anhydride would be more reactive towards the amine group of Tris than the hydroxy group of the solvent (Fessenden and Fessenden 1986). The reaction is outlined in figure 5.3.

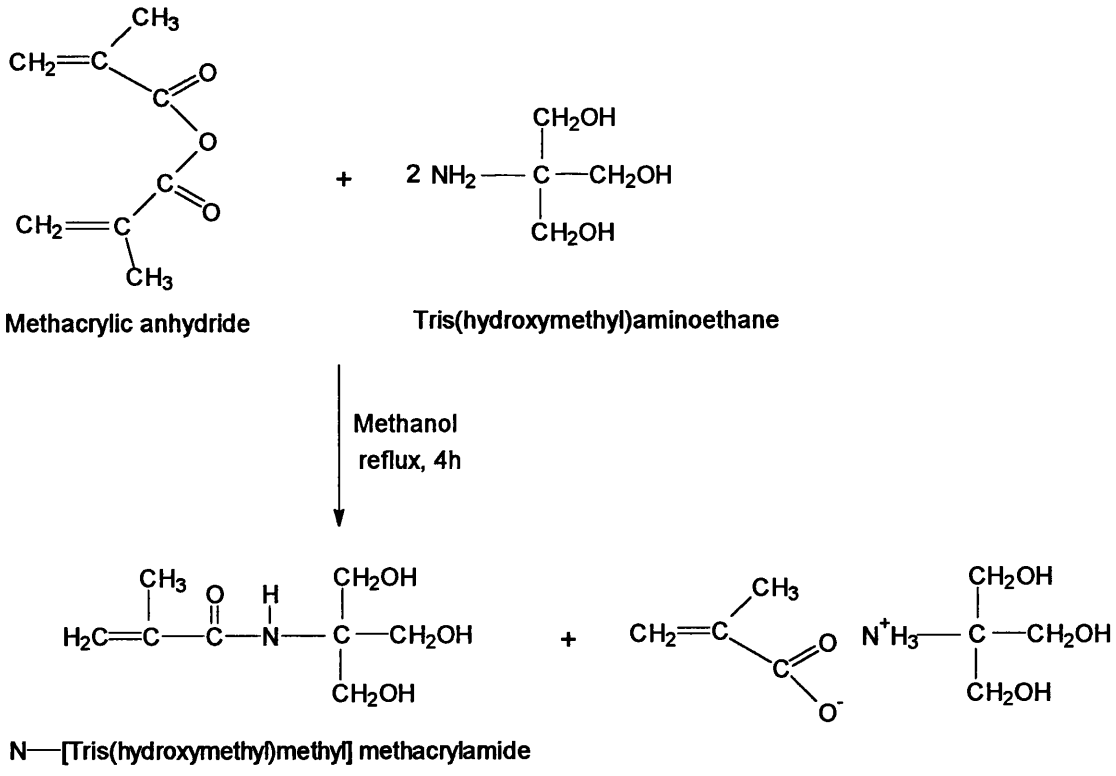


Figure 5.3: *Synthesis of N-[Tris(hydroxymethyl)methyl]methacrylamide.*

The crude monomer yield was reasonably high (~12 g). The problem was that the monomer was usually lost during work up. This was especially true when the crude product was chromatographed on a silica column. Analysis by TLC showed the crude monomer as two spots which were difficult to separate by changing the solvent system. Trituration of the crude monomer produced a colourless oil which crystallised to produce a white solid. Analysis by IR gave a spectrum (figure 5.4) very similar to that obtained for the Tris methacrylate monomer (Jedlinski and Paprotny, 1966). The spectrum shows a very large hydroxyl peak (~3000 cm^{-1}) amide I and II bands at1650 and 1616 cm^{-1} (Cross, 1960).

The molecular weight of the monomer was determined using mass spectroscopy. The largest peak was 190 $gmol^{-1}$ (figure 5.5) which is the molecular weight of N-[Tris(hydroxymethyl)methyl]-methacrylamide but a low yield was obtained (150 mg). Scaling up of the reaction always gave a large amount of crude product (~30 g); however, most of this was lost at the chromatographic step. Occasionally the crude monomer would polymerise in the round bottom flask during solvent removal. The acrylamide homologue of N-[Tris(hydroxymethyl)methyl] methacrylamide is commercially available, whilst the methacrylamide is not. There may be a fundamental reason why the methacrylamide monomer cannot be synthesised in high yields.

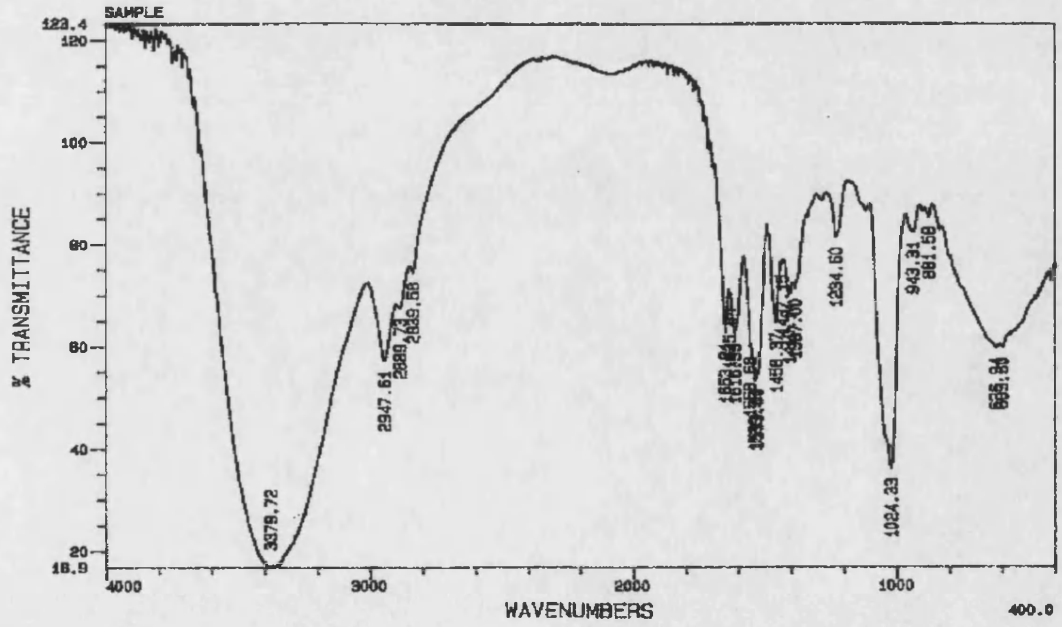


Figure 5.4: *IR spectrum of N-[Tris(hydroxymethyl)methyl]methacrylamide*

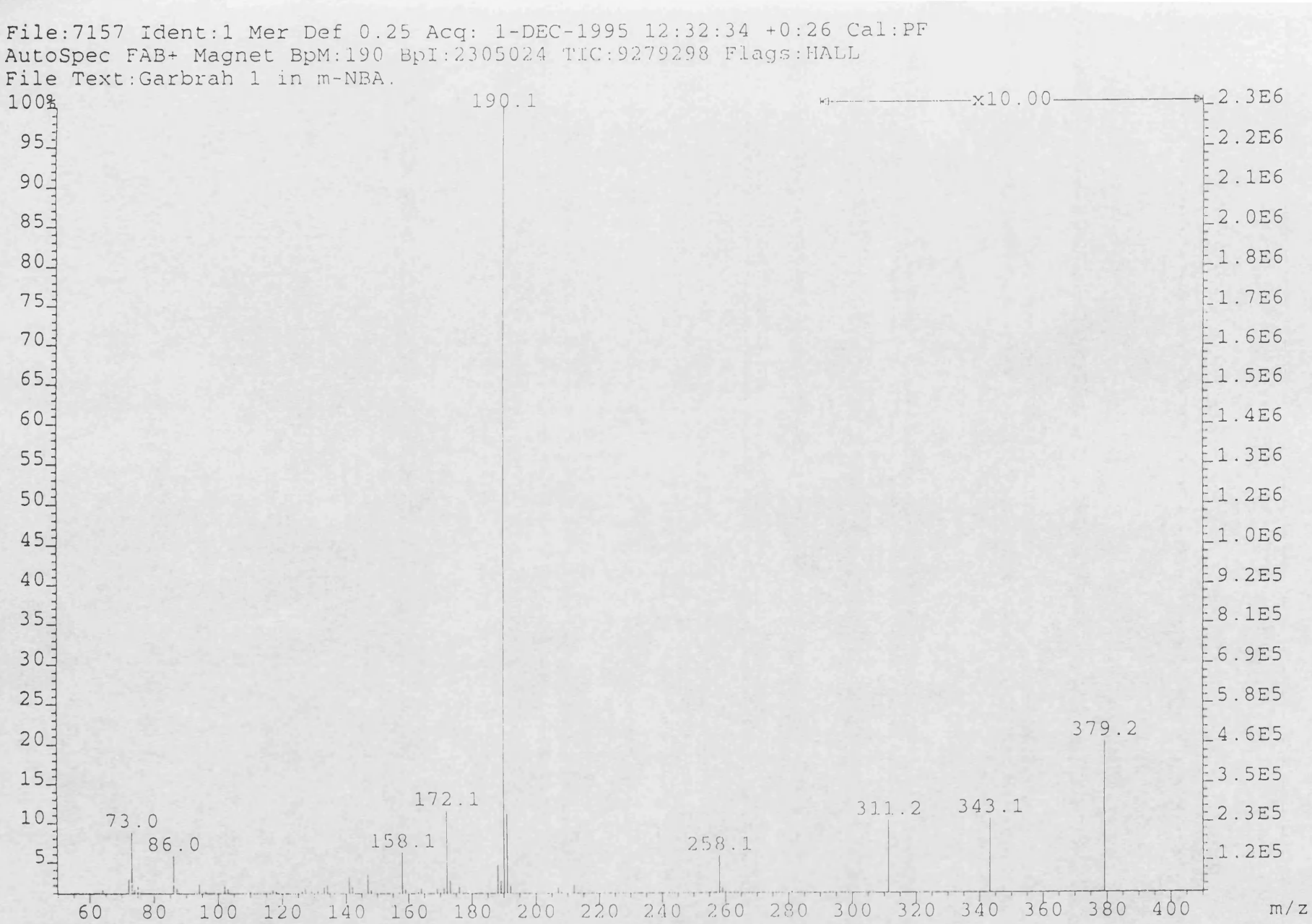


Figure 5.5: *Mass spectrum of N-[Tris(hydroxymethyl)methyl]methacrylamide*

5.4.2. Monomer synthesis incorporating ion exchange purification

The products of reaction between Tris and methacrylic anhydride, should be only the monomer and the ion pair of protonated Tris and methacrylic acid. Under favourable conditions, the ions should be retained by cation or anion exchangers. This procedure however was fruitless because the crude monomer was retained by the columns. This could be due to the ion exchangers (Dowex®) which are made of polystyrene. The monomer may have been retained hydrophobically. Monomer purification was also attempted with a mixed bed ion exchange resin; the crude monomer was also retained by the resin.

5.4.3. Dicyclohexyl carbodiimide mediated coupling of Tris and methacrylic acid

The coupling chemistry of carbodiimides is well known in affinity chromatogaraphy and chemistry (Lowe and Dean, 1974), thus Tris may be coupled to methacrylic acid. This reaction can occur in both aqueous and organic solvents. An organic solvent was preferred because the O-dicyclohexyl urea formed would be easier to remove (figure 5.6) from the monomer. This is because the monomer is water soluble and the O-dicyclohexyl urea is not.

DMF/pyridine (1:1 (v/v)) was used instead of methanol because methacrylic acid could form an ester with methanol. The nmr spectrum (figure 5.7) revealed that the structure is probably the monomer. ^{1}H-NMR (D_2O, δ, ppm): 1.95 (s, 3H, $=CH_3$), 3.6 (s, $(CH_2OH)_3$), 4.85 (s, H_2O), 5.25 (s, cis H), 5.65 (s, trans H), although there are three peaks ; (2.95-3.0, 7.96 ppm) which are due to DMF, which was difficult to remove by rotary evaporation and/or trituration. Tris was used in excess so that all the methacrylic acid would be reacted. Tris was soluble only in hot DMF/pyridine (1:1 (v/v)). Any unreacted Tris would have precipitated out of solution when the reaction medium cooled. One of the main drawbacks of this particular method, was that the O-dicyclohexyl urea was harder to remove than anticipated. The slow addition of water resulted in a thick precipitate of the O-dicyclohexyl urea, most of which could be removed by filtration. A fine precipitate persisted in the filtrate which was difficult to clarify, and scaling up of the reaction exacerbated the problem.

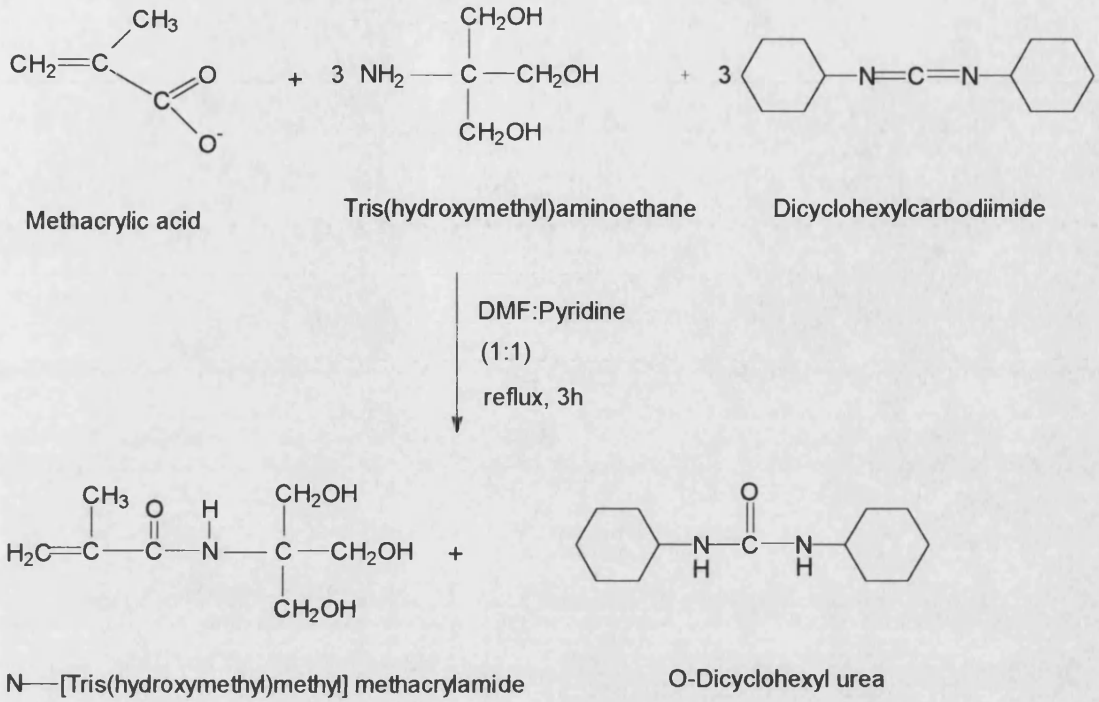
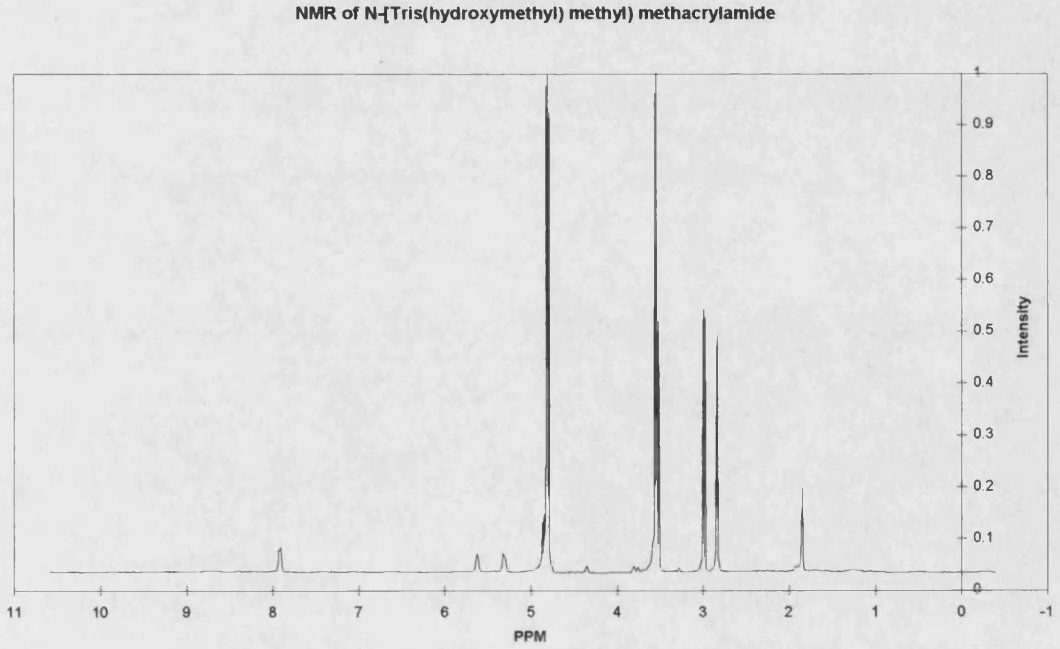


Figure 5.6: *Synthesis of N-[Tris(hydroxymethyl)methyl]methacrylamide via carbodiimide mediated coupling of Tris and methacrylic acid.*

Figure 5.7



5.4.4. Monomer synthesis via saponification

Amides are more resistant to saponification (alkaline hydrolysis) than esters. An attempt was made to exploit this fact by using an excess of methacryloyl chloride to produce an amide with substituted methacryloyl ester groups (figure 5.8). However, prolonged hydrolysis of the molecule could cause the hydrolysis of the amide bond.

A hydrolysis time course did not show an optimum for hydrolysis even after 3 hours. The progress of hydrolysis was followed by using o-toluidine spray for staining, where a purple spot indicated the presence of an amide. The substituted hydroxyl groups would increase the mobility on TLC, as the ester groups hydrolyse the more polar hydroxyl groups reduce the mobility. The results were inconclusive, the distance migrated by the spots did not alter after 3 hours incubation with KOH. A cautious time of 15 minutes was chosen which revealed that the amide bond was present. The pooled product from the silica column did not have the same IR spectrum (figure 5.9), obtained for the Tris monomer in section 5.4.1 (figure 5.4) or that obtained by Paprotny and Jedlinski (1966).

The evidence was conflicting because o-toluidine stains purple for amides; thus any free amine will not be shown by TLC. The pooled fractions all gave strong positive purple spots with the stain indicating the presence of amide. There were some fractions that also contained pyridinium chloride, which is a consequence of pyridine scavenging HCl produced by the substitution of methacryloyl groups. However, the purified product did not have the same properties of the monomer produced by the reaction of methacrylic anhydride and Tris (section 5.4.1).

A possible explanation could be due to the hydrolysis of the three Tris hydroxyl substituted ester groups was not going to completion. This may be a result of the increased stability of the ester groups by virtue of the methyl group on the alpha carbon of the double bond (figure 5.8; Boschetti, 1989). This could explain the difference seen in the IR spectra of the products from the reaction.

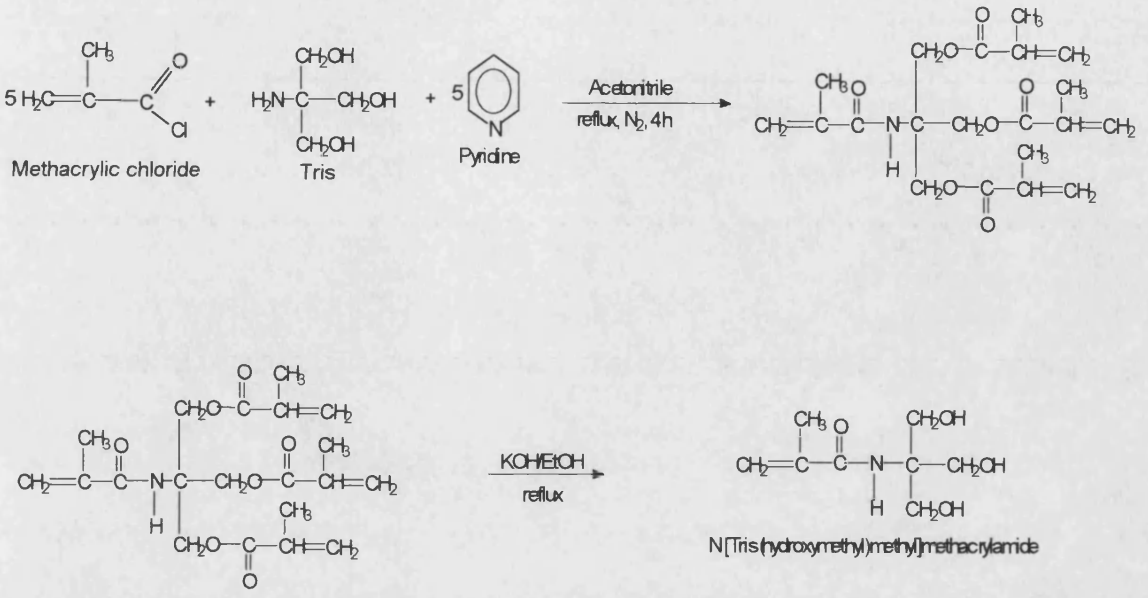


Figure 5.8: *Reaction scheme for saponification of N-[Tris(hydroxymethyl)methyl] methacrylamide*

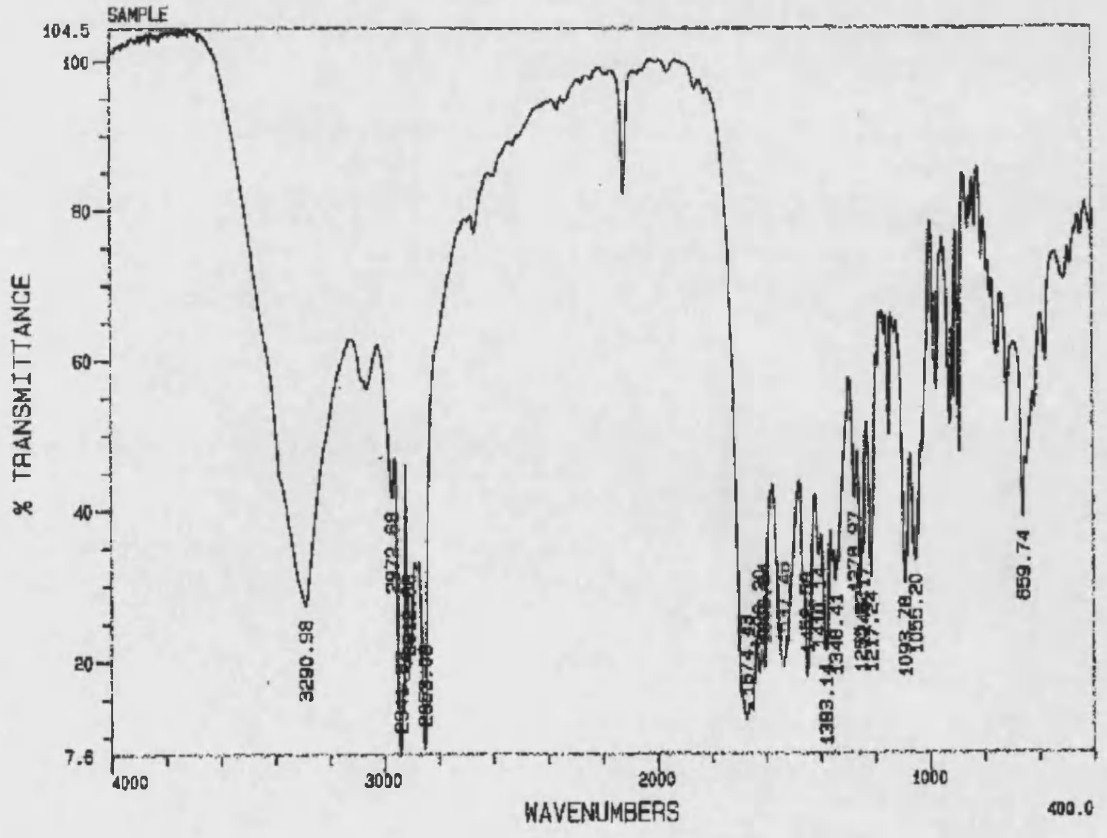


Figure 5.9: *IR spectrum of residue after saponification with methanolic KOH*

5.5. POLYMER SYNTHESIS

5.5.1. Polymerisation of HEMA

The lack of success in synthesising a hydrophilic monomer (sections 5.4.1-5.4.4) meant that commercially available monomers had to be investigated to produce polymers for tentacle affinity chromatography. HEMA was chosen as a monomer for polymerisation because of the presence of a primary hydroxyls and the presence of a methyl group. The methyl group stabilises the ester bond in the HEMA molecule (Boschetti, 1989 and Mazza 1989). This was important in two respects; stability of the polymer during dye ligand substitution and during immobilisation of the polymer to the matrix. These steps require high pH. However, polymerisation was difficult to control and the solution became cloudy usually in about 5 min or even more rapidly in some cases. The cloudiness was due to polyHEMA precipitating. This could not be avoided even if the amount of initiator was increased or the monomer concentration varied (table 5.1). The inhibitor column removal step was omitted from some of the polymerisation attempts, in order to reduce the conversion of monomer to polymer. This also had no effect on the reaction as products were still insoluble (table 5.1). Thus the results tend to suggest that poly HEMA is insoluble even at low monomer conversions. Hence HEMA cannot be used as a basis for tentacle polymer.

Table 5.1: Summary of HEMA polymerisation

Monomer concentration	Initiator concentration	Comments
HEMA 50% v/v	0.5% v/v APS	white precipitate in 5 min
HEMA 50% v/v	0.5% v/v APS	white precipitate in 5 min
HEMA 10% v/v	0.1% v/v APS	white precipitate in 5 min
HEMA 10% v/v *	0.1% v/v APS	white precipitate in 5 min
N-hydroxy methyl acrylamide : HEMA (2:1) 10% v/v*	0.1% v/v APS	white precipitate in 5 min

* No inhibitor column removal step.

5.5.2. Polymerisation of hydroxy monomers

Fuller and Bright (1977) synthesised soluble co-polymers of glycidyl methacrylate and choline (charged monomer), which would introduce ion exchange characteristics into the polymer. Hence neutral monomers were chosen (figure 5.2) which contained primary hydroxyl groups so that the dye could be attached to the polymer. Table 5.2 shows the only viable polymer was the (N-hydroxy methyl)acrylamide-allyl alcohol co-polymer. The other co-polymers or homopolymers formed very faint precipitates with acetone. These results indicate that the homopolymers or copolymers did not polymerise at all, or formed polymers which were insoluble in water or 33% (v/v) ethanol.

This (N-hydroxy methyl)acrylamide-allyl alcohol co-polymer was not ideal because the (N-hydroxy methyl) acrylamide monomer has an alkali labile amide bond, which could be

hydrolysed either during the dye coupling stage or the immobilisation stage of the co-polymer to the support. These two steps require high pH.

Table 5.2: Summary of polymerisation attempts using hydroxyl monomers

Monomer (s)	Amount of monomer (mmole)	Comments
Allyl alcohol	30	No polymerisation
N-(hydroxy methyl) acrylamide	30	Gel formed <10 min
Allyl glycidyl propandiol	30	No polymerisation
Glycidyl methacrylate	30	Gel formed <15 min
Allyl alcohol N-(Hydroxy methyl) acrylamide	15 15	Polymer precipitated in acetone
Glycidyl methacrylate allyl alcohol	15 15	Small amount of polymer precipitated in acetone
Glycidyl methacrylate N-(hydroxy methyl) acrylamide	30 30	Gel formed <10 min
Glycidyl methacrylate N-(hydroxy methyl) acrylamide	7.5 22.5	Gel formed <10 min
Glycidyl methacrylate N-(hydroxy methyl) acrylamide	5 25	Gel formed <10 min
Glycidyl methacrylate N-(hydroxy methyl) acrylamide	2.5 27.5	Gel formed <30 min

5.5.3. Blue (N-hydroxyl methyl) acrylamide-allyl alcohol co-polymer

Cibacron blue was coupled on to the (N-hydroxyl methyl) acrylamide-allyl alcohol co-polymer after 5 cycles of addition (figure 5.10, section 5.3.3). The dye substituted polymer was coupled onto PVA-polystyrene. However, only a small amount of polymer

was immobilised. This was confirmed by the pale blue colour of the beads, and low dye loading (23 nmole ml^{-1}). This result highlighted the fact that even in the presence of primary hydroxyl side chains the polymer could not be immobilised in larger amounts despite a high concentration of polymer in the coupling solution. The reason for this can be attributed to the weaker nucleophilicity of hydroxyls compared to amine or thiol groups (Sundberg and Porath, 1975). The more reactive hydroxyl groups will probably be substituted with dye, leaving the less reactive hydroxyl groups to couple to the gel. There could also be partial hydrolysis of the polymer because the amide bond could be hydrolysed. This would therefore hydrolyse the dye from the polymer reducing the effective dye loading of the polymer (figure 5.11). This may explain the low dye loading observed when the dyed co-polymer was immobilised onto the surface of PVA-polystyrene.

The alkaline instability of the amide bond is one of the reasons why the synthetic polymeric supports such as Biogel® and Trisacryl® have not found widespread use in bioseparations. Until this problem has been fully addressed, these polymeric supports will continue to be overlooked by the down stream purification specialists.

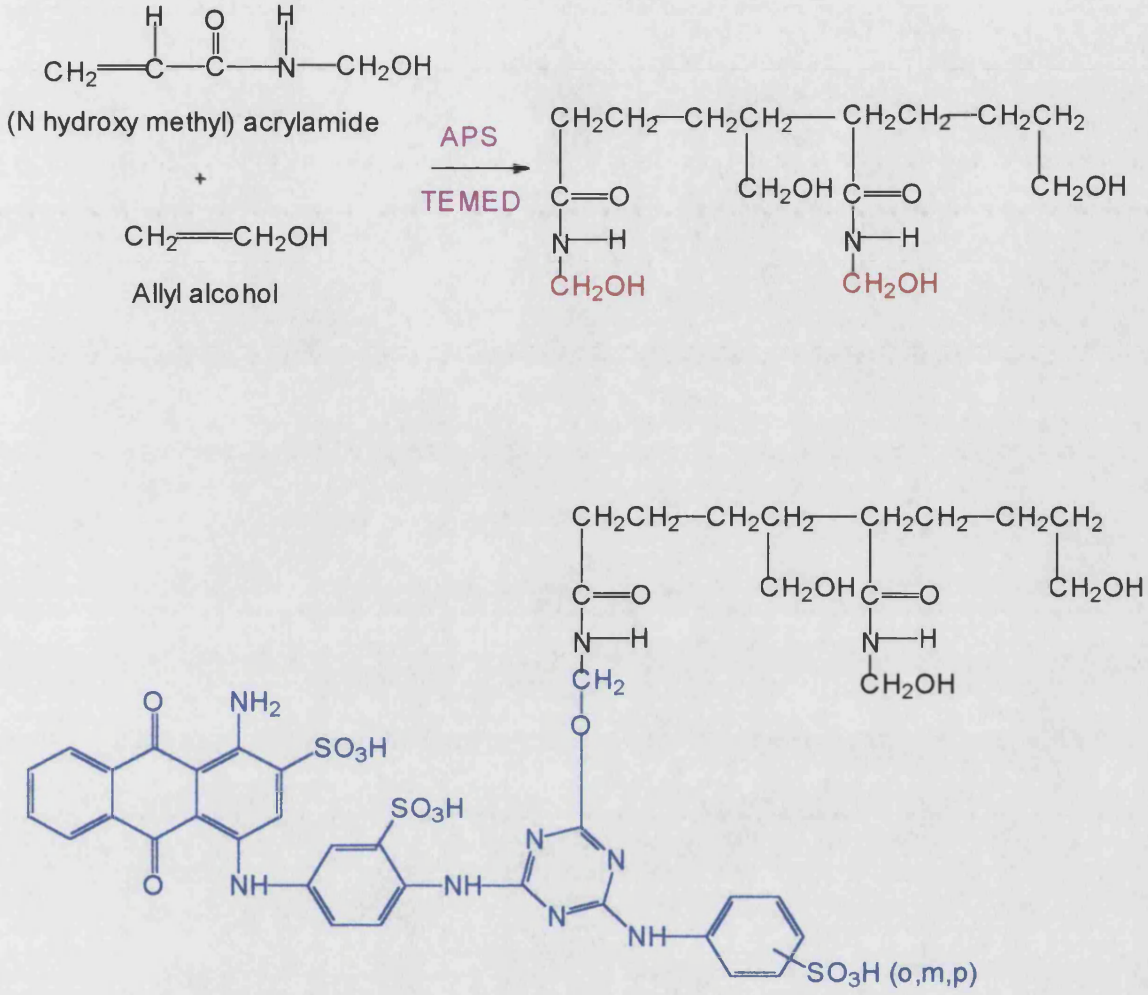


Figure 5.10: *Synthesis of blue N-Hydroxyl methyl) acrylamide-allyl alcohol co-polymer*

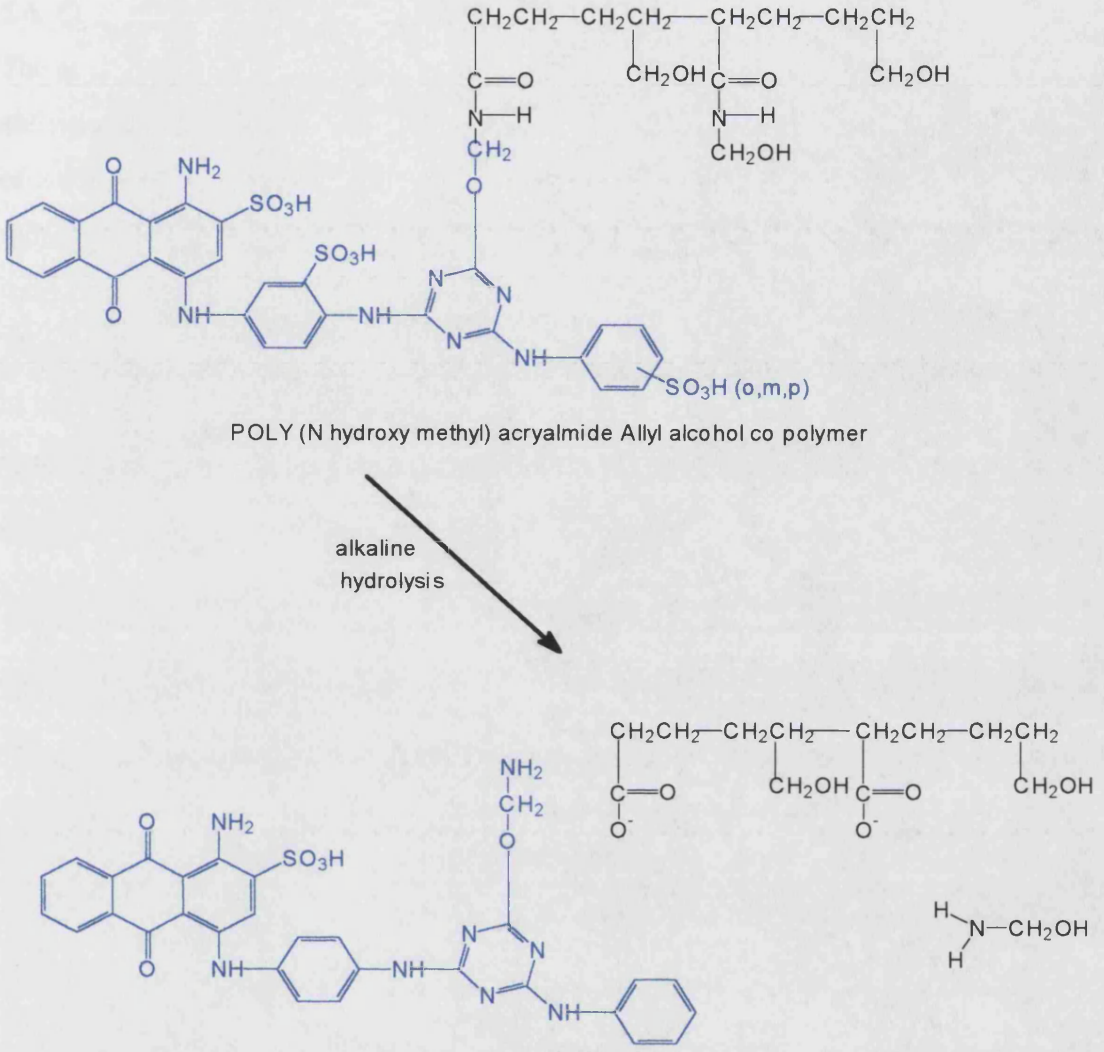


Figure 5.11: *Alkaline hydrolysis of blue N-Hydroxyl methyl) acrylamide-allyl alcohol co-polymer*

5.6. CONCLUSIONS

The synthesis of a hydrophilic monomer (figure 5.3) has posed more difficulties than was anticipated. There appears to be very little work done in this area except for production of a monomer for contact lens material (Friends *et al.*, 1993), and an alkaline resistant monomer used for gel electrophoresis (Chiari *et al.*, 1994; Miertus *et al.*, 1994). These workers reacted acid chlorides with ethanolamine and aminoethoxyethanol respectively. These monomers although suitable would not have been as efficient as the Tris monomer in terms of dye loading and hydrophilicity. A Tris monomer would have three primary hydroxy groups making the monomer extremely hyrophilic, and also a greater number of groups for dye ligand attachment.

The inability to synthesise a monomer was frustrating because Jedlinksi and Paprotny (1968) had polymerised the N-[Tris(hydroxymethyl)methyl]methacrylamide monomer to low conversions (40%) in water but produced soluble polymers of high molecular weight. They also polymerised the same monomer in DMF which did not form a precipitate when polymerised to high conversions. This polymer would have had a large number of hydroxyl group side chains which would have made probably a highly water soluble polymer. A polymer with these properties would have been necessary to prevent the denaturation of biological molecules, and the reduction of non-specific interactions which result in low protein recoveries. This polymer could be easily derivatised with dye, and hence immobilise the polymer, onto the support surface.

Polymer synthesis yielded only one viable polymer; (N-Hydroxyl methyl) acrylamide-allyl alcohol co-polymer. However, this polymer was far from ideal because of the presence of alkali labile bonds. Cibacron blue was immobilised to this polymer however, only a small amount of polymer was immobilised which was probably due to the lack of alkaline stability.

The lack of commercially available primary hydroxyls containing polymers and the lack of published literature indicate that there may well be a fundamental problem in the synthesis of this type of monomer and hence the subsequent polymerisation of the monomer.

CHAPTER SIX

FINAL CONCLUSIONS

PVA-polystyrene beads have been shown to provide a stable support for the synthesis of tentacle affinity supports. The support was synthesised by simply stirring polystyrene beads in an aqueous solution of PVA followed by cross linking in situ. The mechanism of PVA binding to the polystyrene surface is unknown but it may be due to a combination of hydrophobic, Van der Waals and London forces. Terephthalaldehyde proved to be a fortunate choice for cross linkages (Kuhn and Balmer, 1962); 1,3-bromopropanol or epichlorohydrin could have been the alternatives. The strength of the cross linking showed remarkable chemical stability. The PVA layer withstood exposure to 10 M KOH, 2 M NaOH, refluxing in epichlorohydrin (93°C) and repeated heating at 60°C (dye coupling reactions). Solvents such as DMF, acetone and DMSO, had no apparent effect on the cross linkages. The inherent stability of PVA-polystyrene allowed a wide range of activation procedures to be explored.

The aim of the study was to immobilise pre-characterised polymers onto a non-porous support. However, it became apparent that this idea would not be entirely feasible as was demonstrated by the polymers; dextran, HE-starch, PEI, and PVA. These dye-polymer conjugates were immobilised in low amounts (<500 $\mu g\ ml^{-1}$) on the support surface, as a consequence the supports exhibited low protein binding capacities. Immobilised blue-PEI exhibited pH dependent binding and was thought to be due to the anion exchange characteristics of PEI.

A co-polymer of alkyl alcohol and N-(Hydroxy methyl) acrylamide was synthesised. This N-(hydroxy methyl)acrylamide-allyl alcohol co-polymer was far from ideal, given the labile nature of amide bonds (Boschetti, 1989; Fessenden and Fessenden, 1986). The co-polymer was also immobilised in low amounts; this may be due in part to alkaline hydrolysis which liberates dye molecules from the support.

The most successful tentacle support was based on the immobilisation of underivatised dextran followed by the coupling of Cibacron blue. This method increased the amount of polymer immobilised (800 $\mu g\ ml^{-1}$). A combination of weak nucleophilcity of the

hydroxyl groups, low surface area and the high molecular weight (5 x 10^6 Da) may have contributed to the low amount of bound polymer. Nevertheless, a relatively high amount of Cibacron blue was immobilised, some of the dye loadings were even greater than Blue Sepharose CL-6B (table 6.1).

Table 6.1: *The range of blue dextran PVA-polystyrene supports synthesised*

Dye affinity support	**Immobilised dye concentration (μ mole ml^{-1} adsorbent)**
PV1	0.73
PV2	1.2
PV3	6.2
PV4	7
Blue Sepharose CL-6B	4

However, equilibrium binding capacities of the more highly substituted supports (PV3 and PV4) exhibited the lowest capacities; conversely the lowest loadings (PV1 and PV2) exhibited higher capacities. This could be due to an increase in dye-dye interactions, reducing the amount of ligands available for protein binding. Thus a lower ligand concentration is optimal for the tentacle affinity support. An added advantage is that the low ligand concentration reduces the risk of ligand leakage which is an important factor in downstream processing.

Analysis of the kinetic and equilibrium data, showed the value of K_d was of the order of 10^{-7} M irrespective of dye loading. The binding capacity of the supports decreased with increased dye loading. The reason for this reduction in capacity is most likely due to the number of intramolecular dye-dye interactions increasing thus reducing the availability of

the dye molecules. These values of K_d are similar to that of obtained for blue Sepharose (Liu and Stellwagen, 1987).

The tentacle configuration increases the "effective surface area" of PVA-polystyrene. Resulting in a 14 fold increase in protein binding capacity when compared to the "theoretical" capacity due to surface area. The ligand utilisation was however disappointing. The lowest dye loading (PV1) had a utilisation of approximately 7%. The other loadings were 2% (PV2), 0.1% (PV3 and PV4) which were particularly low when compared to Blue Sepharose which displayed a ligand utilisation of approximately 2%. The lowest loading (PV1) displayed a respectable ligand utilisation, most of conventional porous adsorbents rarely have ligand utilisation values above 3%.

The non-porous tentacle support exhibited fast binding kinetics, with equilibrium being acheived five times faster than porous blue Sepharose CL-6B. Protein binding is limited to the external surface, thus mass transfer limitations are greatly reduced. Thus the cycle times for adsorption, washing and elution and regeneration would be reduced.

Dextran PVA-polystyrene also displayed low non-specific binding (40 $\mu g\ ml^{-1}$) which is very important for an affinity matrix. Thus all the observed protein binding is due to the immobilised ligand and the eluted target molecule should be of high purity.

The tentacle support displays a linear relationship between linear flowrate and back pressure, and displays the characteristics of a "rigid" bead (Stewart *et al.*, 1992). A linear flowrate of 1200 cm h^{-1} produced a back pressure of only 0.06M Pa. In contrast for Sepharose CL-6B a linear flowrate of only 350 cm h^{-1} produced a back pressure of 0.2 MPa. This suggests that the tentacle support is suitable for use in either high or intermediate pressure chromatography, which would be suitable for large process scale applications.

The purification of fumarase was not successful. The enzyme did not interact strongly with the tentacle support, most of the enzyme passed through the column unbound. The "purified" fumarase could only be visualised on SDS-PAGE after concentration with PEG. However there was an increase in specific activity, despite the low purity.

PVA-polystyrene is a useful starting material with which to synthesise tentacle affinity supports. The support is chemically and physically stable and this stability allows a wide range of activation possibilties to be explored. Blue dextran PVA-polystyrene, has proved that the tentacle affinity support is a highly feasible option, available to the downstream processing specialist.

Future work

To have a better idea of the blue dextran PVA-polystyrene's resolving power, a crude enzyme requiring an adenyl co-factor would be a better choice of protein to purify, since the dye's interaction with such proteins are well known and characterised. The possibility of using polymer shielding could also be investigated where polymers such as poly(vinylpyrroldone) or poly(N-vinyl caprolactam) (Galaev and Mattiasson, 1993) are used to pre-coat a dye affinity column via multi-point attachment interactions. The polymer reduces the amount of non-specific binding proteins and enhances the specific binding of the target molecule, to such an extent that the target protein is eluted to a high level of purity (Galaev and Mattiasson, 1993; Garg *et al*, 1996).

The yield of alkali stable monomer could be improved by the presence of polymerisation inhibitors during the purification. The subsequent polymer would have a large number of hydroxyl group side chains, for immobilisation of dye and attachment to the bead surface. The monomer could be co-grafted with a dye monomer onto the PVA-polystyrene surface using the method of Gonen and Kohn (1981), thus producing a tentacle support with grafted polymer tentacles, removing the problems of polymer immobilisation.

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